A NEW BROAD SPECTRUM DISINFECTANT SUITABLE FOR THE FOOD INDUSTRY

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by
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DEDICATION

This work is dedicated to my parents Salvador and Margarita Aita and to my brothers Salvador Aita and Giorgio Aita. Their love, support and encouragement throughout these years have allowed me to achieve and complete the goals in my life. This dedication is also extended to my husband Dr. Marcio S. DeQueiroz and to our daughter Alexis. Without them, this research would not have been accomplished, and I would not be.

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LIST OF ABREVIATIONS

Abbreviation	Term
ANOVA	Analysis of variance
ARTCA	Antimicrobial Reform Technical Corrections Act
ATCC	American type culture collection
ATP	Adenosine triphosphate
alg	Alginate
cal	Calories
CFU	Colony forming units
CFR	Code of federal regulations
CFSAN	Center for food safety and applied nutrition
Cl_2	Chlorine
$Cl^{\bullet}$	Chlorine radical
ClO_3^-	Chlorate ion
cm	Centimeter
CSLM	Confocal scanning laser microscopy
Da	Dalton
DMPO	5, 5, dimethyl-1-pyrroline-N-oxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EMB	Eosin methylene blue
EPA	Environmental protection agency
EPS	Extracellular polymeric substances
FDA	Food and drug administration
FFDCA	Federal food, drug, and cosmetic Act
FIC	Fractional inhibitory concentration
FIFRA	Federal insecticide, fungicide, and rodenticide act
FQPA	Food Quality Protection Act
FSIS	Food Safety and Inspection Service
FTIR	Fourier transform infrared
g	Gram
Gal	Gallon
h	Hour
HACCP	Hazard Analysis Critical Control Points
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HOO^-	Perhydroxyl ions
in	Inch
L	Liter
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
M	Molar
mg	Milligram

min	Minute
µg	Microgram
µl	Microliter
ml	Milliliter
mm	Millimiter
mM	Millimolar
µs	Microsecond
ms	Millisecond
mol	Mole
N	Normal
NaOCl	Sodium hypochlorite
nm	Nanometer
$^{1}O_2$	Singlet oxygen
$^{3}O_2$	Triplet oxygen
O_2^-	Superoxide
OCl^-	Hypochlorite ion
OCl_2^-	Chlorite ion
OH^-	Hydroxyl ion
$OH^\bullet$	Hydroxyl radical
PCA	Plate count agar
ppm	Parts per million
PMF	Proton motive force
QAC	Quaternary ammonium compound
$RO^\bullet$	Alkoxyl radical
$RO_2^\bullet$	Peroxyl radical
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
s	Second
SAS	Statistical analysis software
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
t-MVP	Trans-1 (2’-methoxyvinyl) pyrine
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSP	Trisodium phosphate
Vol	Volume
w	Weight
YE	Yeast extract

ABSTRACT

A unique biocide composition (patent pending) that is formed from a hydrogen peroxide and sodium hypochlorite mixture was investigated. A biocidal “complex” is formed by adding the peroxide to the hypochlorite in an amount so that the weight ratio of the peroxide to the hypochlorite is no less than 1:10. The chemical structure of this biocidal “complex” is uncertain but we postulate that it is a semi-stable complex, whose stability is disrupted by heat, acid, U/V exposure and the presence of organic matter (i.e., microbes) The antimicrobial activity of the biocidal “complex” is most likely a combination effect between oxidation and reductive mechanisms The biocidal complex needed from one sixth to one half the concentration of hydrogen peroxide and from one twentieth to one half that of sodium hypochlorite to kill a range of Gram-positive and Gram-negative cells. In the case of bacterial spores (*Bacillus* sp.), MICs of the biocidal complex ranged from one twentieth to one half and from one fourth to one half for hydrogen peroxide and sodium hypochlorite, respectively. FIC values for both bacterial cells and spores were less than one. FIC values of less than one indicate that a synergistic effect exists between biocide components. The activity of the biocide is stable at alkaline pH, with a half-life of at least 42 days. It is non-corrosive and can be effective in both a dip and spray mode against bacterial cells in their planktonic or sessile state. Our studies indicate that sodium hypochlorite is not only synergistic with hydrogen peroxide but with sodium peroxide as well The use of this biocidal complex may provide a safe, effective and easy method for killing potential pathogens as well as for disinfecting and removing biofilms, as they pose a threat to human safety, particularly in the Food Industry.

INTRODUCTION

Humans are constantly exposed to microorganisms (e.g., bacteria, fungi, algae, viruses) and microbial entities (e.g., prions). Microbes are ubiquitous, and under any growth condition where life can exist, and even in extreme environments, they will grow and in some instances may pose a risk to human health. Minimization of microbial populations and microbial entities under non-sterile conditions generally requires the use of antimicrobial agents.

Both physical and chemical antimicrobial agents are widely used as preservatives in many types of pharmaceuticals, cosmetics and foods and other industrial products. Antimicrobials can be disinfectants or sanitizers. Disinfectants and sanitizers have different effects on a variety of microorganisms and microbial entities (Russell, 1983). Bacteria show varying degrees of resistance towards disinfectants. Prions are the most resistant of all microbial entities to antimicrobial agents. Bacterial spores and mycobacteria are the most resistant forms of the bacteria, followed by Gram-negative bacteria, which are generally more resistant than vegetative Gram-positive bacteria such as the staphylococci and enterococci (Morton et al., 1998).

Resistance to antimicrobial agents can also be a function of formation of slimes and/or biofilms. All microorganisms can form biofilms upon adhesion to surfaces. Natural biofilms develop into ecosystems with wide varieties of microorganisms embedded in polysaccharide matrices, which act as a penetration barrier for active components of antimicrobials (Heinzel, 1998). It is necessary to break up or destabilize biofilms in order to make these microorganisms accessible to most antimicrobials.

Hydrogen peroxide (H_2O_2) and sodium hypochlorite (NaClO) are both food grade antimicrobials (depending on concentration), widely used independently, or in combination with

peracetic acid (Palop et al, 1998), methanol (Waites, 1985) or formaldehyde (Alasri et al., 1992). Hydrogen peroxide has the ability to generate radicals, whereas the active agent in hypochlorite compounds is undissociated hypochlorous acid (Rutala and Weber, 1997). In combination, these compounds generate singlet oxygen, a powerful oxidant and antimicrobial, as well as other forms of oxidizing compounds such as hydroxyl radicals and superoxide radicals.

This investigation focused on the antimicrobial applications of a combination of hydrogen peroxide and sodium hypochlorite complex as a disinfectant, sanitizer and/or sporicide against both bacterial spores and vegetative cells in planktonic form or as biofilms.

1. LITERATURE REVIEW

1.1. Definition of Antimicrobial Agents

Antimicrobial agents may be either physical conditions or chemical agents or combinations of both. Physical antimicrobial agents include techniques such as the use of moist heat, dry heat, ultra violet and ionizing radiations, and hydrostatic pressure to reduce microbial counts or to achieve commercial sterilization (Block, 1991). Chemical agents are used as chemotherapeutic agents, antiseptics, food preservatives, sanitizers, disinfectants or sterilizers (sporicides) (Block, 1991). Chemotherapeutic agents such as antibiotics are used in the treatment of diseases of man, animals and plants. In addition, low doses of antibiotics are applied to protect crops, and given to healthy animals, including farm-raised fish, to make them grow faster with less feed. Antiseptics are substances applied topically to living tissues to prevent or arrest the growth or action of microorganisms. Food preservatives are chemical compounds used in foods, alone or in conjunction with physical agents, to control microflora as well as to extend shelf life and to ensure the safety of food products. A sanitizer is defined as an agent added to an inanimate object (food contact or non-food contact products) to reduce, but not necessarily to eliminate, the number of bacterial contaminants to levels that are considered safe by public health codes or regulations. Disinfectants are chemical or physical agents applied to inanimate objects to destroy or irreversibly inactivate disease causing microorganisms, but not necessarily their spores. Sterilizing agents, on the other hand, are chemical or physical agents used to destroy or eliminate all forms of microbial life, including spores of *Bacillus subtilis* (Sagripanti and Bonifacino, 1996).

Biocide is a general term used to denote a chemical that possesses antiseptic, disinfectant or preservative activity (Block, 1991). In the United States, biocidal products are usually referred

to as antimicrobial pesticides. According to the USA legislation, antimicrobial pesticides, such as disinfectant, sanitizers and sterilizers, are pesticides intended to: (1) disinfect, sanitize, reduce or mitigate growth or development of microbiological organisms or (2) protect inanimate objects (e.g. floors and walls), industrial processes or systems, surfaces or other chemical substances from contamination or deterioration caused by bacteria, viruses, fungi, protozoa, algae or slime (FDA, 2003). This definition does not include certain antimicrobial pesticides intended for food use, nor does apply to personal health care disinfectants, which are regulated as "drugs" under the Food and Drug Administration (FDA).

1.2. Classification of Chemical Sporicides, Disinfectant and Sanitizer Agents

Biocides vary in their chemical structures and also in their mode of action. However, the final damage, when lethal concentrations are used, may show similarities. Biocides must reach and interact with their microbial target site(s) to be effective. Although most biocides are non-specific, a compound such as 5-chloro-2-(2, 4 –dochlorophenoxy) phenol (triclosan) has been found to block lipid synthesis by targeting the enol-acyl carrier protein reductase (McMurray et al., 1999). Other biocide target sites may include outer cellular components, the cytoplasmic membrane, cytoplasmic constituents and/or metabolic processes. A summary on biocide targets and effects of some common disinfectants, sporicides and sanitizers is given in Table 1.

1.2.1. Acids and Esters. Both organic (benzoic, citric and sorbic) and inorganic acids (sulphuric, hydrochloric, phosphoric, and sulfamic) are active at low pH, particularly in their un-dissociated form (Russell, 1990). They are effective against bacteria, some viruses and fungi and are often formulated in combination with other disinfectants (Knight and Cooke, 2002). Antimicrobial activity of organic and inorganic acids involves interference with the cellular uptake of substrate molecules by disruption of tertiary and quaternary protein structures and membranes. The benzoates are known to interfere at the stage of spore germination (Jay, 1992). Limitations

Table 1. Antimicrobial targets, mechanism of interactions and antimicrobial effects of selected biocides (Block 1991, Denyer and Stewart 1998).

Mechanism of Interaction	Antimicrobial Agent	Antimicrobial Targets	Antimicrobial Effect
Halogenation	Hypochlorites chlorine-releasing agents	Amino groups in proteins	Metabolic inhibition
Free-radical oxidation	Peroxygens	Enzyme and protein thiol groups	Metabolic inhibition
Electrostatic (ionic) interaction w/ phospholipids	QAC's, chlorhexidine, polyhexamethylene biguanides	Cell membrane integrity, membrane-bound enzyme ennvironment and function	Leakage, respiratory inhibition, protoplast lysis, effect on PMF intracellular coagulation ATPase inhibition
Penetration/partition into phospholipid bilayer, displacement of phospholipid molecules	Phenols, weak acids parabens	Transmembrane pH gradient, membrane integrity	Leakage, disruption of transport, respiratory and energy coupling processes Possibly cell lysis
Solution of phospholipids	Aliphatic alcohols	Membrane integrity	Leakage
Membrane protein solubilization	Anionic surfactants Antifungal imidazoles	Cell membrane integrity, membrane bound enzyme, environment and function	Leakage, uncoupling of energy processes, lysis inhibit ergosterol synthesis, induce gross membrane damage

Table 1 cont.

Mechanism of Interaction	Antimicrobial Agent	Antimicrobial Targets	Antimicrobial Effect
Oxidation of thiol groups	Izothiazolinones, organomercurials hypochlorites organochlorine derivatives heavy metal salts oxides, bronopol	Thiol containing cytoplasmic membrane bound enzymes	Metabolic inhibition
General alkylation reactions	Glutaraldehyde formaldehyde, oxides chloroacetamide	Biomolecules (DNA, proteins, RNA) containing amino,imino, amide, carboxyl and thiol groups	Metabollic and replicative inhibition Cell wall damage may occur by interaction with NH_2 groups
Metal ion chelation	EDTA, oxines	Divalent cation-mediated outer membrane integrity, Gram negative cell wall principle target, metal iron requiring enzyme processes	Leakage, increased susceptibility to applied stress Induce release of LPS Metabolic inhibition

LPS= Lipopolysaccharide

to their use include corrosiveness and safety requirements. Esters of p-hydroxybenzoic acid (parabens) are widely used as preservatives (Russel, 1990). The parabens are active over the pH range of 4 to 8 and are more effective against molds than against yeasts (Jay, 1992). Antimicrobial properties involve disruption of the proton motive force (PMF) (Maillard, 2002).

1.2.2. Acid-anionic Surfactants. Dodecyl benzene sulfonic acid, dodecyl benzene sulfonic acid sodium salt and naphthalene sulfonic acid are typical acid-anionic surfactants. Antimicrobial activities of these compounds are greater at low pH and they are more effective against Gram-positive than Gram-negative bacteria. They have both a hydrophilic and a hydrophobic domain which facilitate their interaction with cell membranes. The hydrophilic domain carries a negative charge at pHs above its pKa. These groups may include carboxylic acid, sulfonic acid, sulfuric acid ester, phosphoric acid ester or phosphoric acid. The hydrophobic domain is made of straight or branched alkyl chains. The antimicrobial activity is related to the length of the alkyl chain. Maximum antimicrobial activity is observed in surfactants with alkyl chains of 12 carbon atoms. The antimicrobial properties of acid-anionic surfactants are due to one or more of the following events: disorganization of the cell membranes, inhibition of key enzymes, interruption of cellular transport or denaturation of cellular proteins (Block, 1991).

1.2.3. Alcohols. Ethanol and isopropyl alcohol at concentrations ranging from 60-70% are lethal to nonsporing bacteria but have no sporicidal activity (Knight and Cooke, 2002). However, the addition of 1% sodium or potassium hydroxide, acids, or 10% amyl-m-cresol to 70% alcohol has been reported to enhance sporicidal activity (Russel, 1999). The antimicrobial properties of alcohols are due to denaturation of proteins. However, secondary effects with ethanol such as inhibition of nucleic acid, protein and peptidoglycan synthesis have been observed in *E. coli* cells (Maillard, 2002).

1.2.4. Aldehydes. Glutaraldehyde and formaldehyde are powerful antimicrobials with a broad spectrum of activity against many microorganisms. Formaldehyde is both bactericidal and sporicidal but at a much slower rate than glutaraldehyde. Their activity is affected by pH, with alkaline conditions being more effective than acid ones (McDonnel and Russell, 1999). Possible targets include biomolecules (e.g., proteins, RNA, DNA) containing amino, imino, and amide groups which result in metabolic inhibition and possibly cell wall damage (Denyer and Stewart, 1998). In Gram-negative bacteria, glutaraldehyde interacts primarily with outer components of the cell such as lipoproteins (Maillard, 2002).

1.2.5. Biguanides. Chlorhexidine is the most important biguanide and is used as either the acetate or gluconate salt (McDonnel and Russell, 1999). It has a broad spectrum of activity, being an effective bacteriostatic and bactericidal agent against Gram-positive and Gram-negative microorganisms. It is sporostatic rather than sporicidal; however, it becomes sporicidal at elevated temperatures. The antimicrobial activity of chlorhexidine is greater between pH 5 and 7 but it is inactivated in the presence of organic matter (Knight and Cooke, 2002). Lethality of biguanides is attributed to damage to the cytoplasmic membrane integrity (Block, 1991). Biguanides cause a high rate of leakage of intracellular components and higher concentrations result in coagulation of the cytosol (Maillard, 2002). A secondary target of biguanides is the enzyme adenosine triphosphatase (Maillard, 2002).

1.2.6. Chlorine Active Compounds. Chlorine compounds can be considered to be of three types: chlorine gas, hypochlorites (e.g., sodium, calcium, lithium and potassium) and chlorine releasing agents (trichloroisocyanuric acid, sodium dichloroisocyanurate, dichlorodimethyl hydantoin, chloramines T) (McDonnel and Russell, 1999 and Knight and Cooke, 2002). Chlorine gas can be used for the disinfection of water, but sodium hypochlorite and sodium

dichloroisocyanurate are the agents of choice. Chlorine compounds are bactericidal and sporicidal. Their activity is related to their solubility, amount of available chlorine present and pH of the solution. However, their activity is impaired by the presence of organic matter. Hypochlorites are powerful oxidants and can induce lysis in Gram-negative bacteria by affecting the cell wall (Maillard, 2002). A detailed description on the antimicrobial properties of hypochlorites is discussed later.

1.2.7. Iodine and Iodophores. Iodophores contain elemental iodine complexed with chemicals such as surfactants for solubility and phosphoric acid for stability. Iodine and iodophores are effective bactericidal and sporicidal agents. Neutral and low pH favors activity of iodine by allowing the formation of diatomic iodine and hypoiodous acid. Iodophores, on the other hand, are active over a wide pH range (McDonel and Russell, 1999). As a microbicide, iodine reacts with (1) N-H functions of amino acids and the bases of the nucleotides disrupting protein and nucleic acid structures, (2) the S-H group in the amino acid cysteine thus affecting protein synthesis and (3) carbon-carbon double bonds of unsaturated fatty acids which results in physical changes of lipids and membrane immobilization (Block, 1991).

1.2.8. Nitrogen Compounds. Putative formaldehyde-releasing agents, nitriles, nitrites, pyridines, thiazoles/imidazoles, nitros, amines, anilides and quinolines are the major categories of nitrogen-containing antimicrobials. Sodium azide is the simplest, with antimicrobial properties against Gram-negative bacteria. Putative formaldehyde-releasing agents such as hexamethylenetetramine, polyoxymethylurea, noxythiolin and taurolin are effective against Gram-positive and Gram-negative bacteria. Nitrites are widely used in the meat curing process and are often added in conjunction with sodium erythorbate or sodium ascorbate. Nitriles are highly reactive at low levels but their activity is compromised at alkaline pH. Effectiveness of

some nitrile-derived compounds is claimed against bacteria, fungi and algae associated with the deterioration of paint and adhesives. Pyridine derivatives have been widely used as preservatives, antiseptics and in chemotherapy. Some thiazole/imidazole derivatives exhibit fungicidal properties while others are active against both Gram-positive and Gram-negative bacteria. Mercaptobenzothiazole has both antifungal and antibacterial properties, attributed to its chelating characteristics. Bronopol (2-bromo-2-nitropropanol-1, 3-diol), a nitro derivative, has widespread application in pharmaceutical products, toiletries, consumer products, cooling towers and metalworking fluids. Antimicrobial properties of bronopol include oxidation of thiol groups, with increased activity at pH 8 or 9 over neutral or acidic pHs. Amine derivatives encompass a large and diverse group of compounds with unspecified modes of action that secondarily act as wetting agents and corrosion inhibitors. Derivatives of salicylanilide and carbanilide are widely used in leather, paper and plastics as fungicides. Oxine (8-hydroxyquinoline) is one of the most widely used quinoline derivatives with antifungal, antibacterial, and chelating properties (Block, 1991).

1.2.9. Organomercury Compounds. Organomercury compounds such as phenylmercuric nitrate, phenylmercuric acid and thiomersal (menthiolate) are widely used as preservatives in pharmaceutical products as well as in paints and coatings (Block, 1991 and Russell, 1990). These compounds are bacteriostatic, bactericidal, fungicidal and sporostatic at low concentrations. Sporicidal activity is observed at high temperatures (Block 1991 and 1990).

1.2.10. Peroxygens. Hydrogen peroxide and peracetic acid are both bactericidal and sporicidal. The antimicrobial activity of hydrogen peroxide is due to the generation of hydroxyl radicals. Hydroxyl radicals are a type of reactive oxygen species (ROS). ROS are physiological reactants, some act as signaling molecules; but their overproduction can lead to biological damage. The

term ROS comprises oxygen radicals (e.g., superoxide, O_2^-; peroxyl radical, ROO$^\bullet$; alkoxyl radical, RO$^\bullet$) as well as some oxidants (e.g., ozone, singlet oxygen, hydrogen peroxide, sodium hypochlorite, peroxynitrite) that may be converted to radicals (Hayes et. al., 1997). A radical is an atom or group of atoms with one or more unpaired electrons that have a positive, negative or zero charge (Hayes et. al., 1997). Decomposition of peroxides is increased by metals, light, agitation, metallic salts, catalase and heat. However, a slight excess of acid helps to stabilize this compound. Peracetic acid is considered to be a more potent sporicide than hydrogen peroxide. The activity of peracetic acid is affected slightly by the presence of organic matter and is unaffected by catalase (Russel, 1999).

1.2.11. Phenols and Cresols. Phenols and cresols are poorly sporicidal but inhibit spore germination. The sporicidal activity of these compounds is greatly enhanced at elevated temperatures (Russell, 1999). At low concentrations, inactivation of essential enzymes is observed. However, at high concentrations, these compounds penetrate and disrupt the cell wall and precipitate cell wall proteins (Block, 1991). Low concentrations of phenols have been shown to lyse growing cells of *E. coli*, streptococci and staphylococci (Maillard, 2002).

1.2.12. Quaternary Ammonium Compounds (QACs). The QACs are derived from ammonium salts by replacing the hydrogen atoms with alkyl groups. The chain length of alkyl groups has an effect on the antimicrobial properties of the molecule. The highest antimicrobial activity is shown with chain lengths between 11 and 17 carbons. Little or no antimicrobial activity is observed with chain lengths higher than 20 carbons. QACs are bactericidal against non-mycobacterial, non-sporeforming, Gram-positive bacteria and are sporostatic at low concentrations. They are poorly active against Gram-negative bacteria and are not sporicidal. The long alkyl chains of QACs penetrate between and disrupt phospholipid molecules found

within the cell membranes, causing them to leak. The degree of leakage is higher for Gram-positive than for Gram-negative bacteria (Maillard, 2002). QACs may also denature enzymes vital for growth (McDonnel and Russell 1999, and Knight and Cooke, 2002).

1.2.13. Oxides. Ethylene oxide (know as epoxyethane or dimethylene) and propylene oxide (known as epoxy propane) are the two most important disinfectants. The oxides exist as gases and are soluble in water, oils, rubber and most organic solvents. These compounds react with amines, organic acids and amides. The antimicrobial activity of oxides has been attributed to alkylation of sulfhydryl, amino, carboxyl, phenolic and hydroxyl groups in spores or vegetative cells. Alkylation of nucleic acids with ethylene oxide has been reported as the primary cause of sporicidal and bactericidal activity. Ethylene oxide is also effective against fungi and viruses. The antimicrobial properties of oxides are dependant on factors such as concentration, time exposure, temperature and water vapor (Block, 1991).

1.2.14. Ozone. Ozone is a powerful oxidizing agent and can be found in both the gas and liquid phases. Ozone is highly unstable. It needs to be generated in situ for disinfection and sterilization. Ozone is an effective bactericide, sporicide and virucide. Ozone has also been reported to be effective against fungi and protozoa (Block, 1991). Bacterial cell surfaces are the primary target of ozone. Double bonds of unsaturated lipids within the cell membrane are the primary site of attack. Ozone also reacts with amino acids and modifies purine and pyrimidine bases (Maillard, 2002).

1.3. Regulation of Antimicrobial Agents in the United States

In the United States (U.S.), regulations of pesticides, including antimicrobial agents and agricultural products for plant protection, are dictated by the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Federal Food, Drug and Cosmetic Act (FFDCA). FIFRA and

FFDCA are handled by the Center for Food Safety and Applied Nutrition (CFSAN). FIFRA and FFDCA have been modified on several occasions, most recently by the Food Quality Protection Act (FQPA) of 1996 (Public Law 104-170, 1996) and by the Antimicrobial Reform Technical Corrections Act (ARTCA) of 1998 (Public Law 105-324, 1998).

FIFRA provides the basis for the regulation, sale and distribution of pesticides in the U.S. and is overseen by the Environmental Protection Agency (EPA). FIFRA authorizes the EPA to: (1) register or license (FIFRA §136(a)) pesticides; (2) re-register or re-evaluate pesticides registered prior to November 1, 1984 (FIFRA §136(a)(1)); (3) regulate pesticide imports and exports (FIFRA §136o); (4) suspend or cancel experimental use permits (FIFRA §136(c)) or the registration of any pesticide (FIFRA §136(d)), (5) oversee all aspects of the sale, distribution and use of pesticides (FIFRA §136(j)) including their labeling (FIFRA §136(a)(c)(1)(c)); (6) evaluate pesticides every 15 years (§136(a)(g)); (7) impose post registration obligations (§136(d)(a)(2)) and (8) generate additional data to support continued registration (§136a(c)(2)(B)). FIFRA is administered by EPA's Office of Pesticide Programs (OPP), which also handles all antimicrobial registrations and coordinates safety programs regulating everything from the types of pesticides used to safe levels of exposure for workers.

FFDCA is administered in part by the EPA and in part by the Food and Drug Administration (FDA) and provides the basis for the regulation of pesticides and uses that may result in contact of the pesticide with foods. FDA not only sets forth the requirements for the establishment of legal pesticide tolerances or exemptions (FFDAC § 346(a)) but also regulates the use of pesticides particularly antimicrobial agents that are used in foods as food additives or that come in contact with foods or feed crops (fruits, vegetables and seafood). The U.S.

Department of Agriculture (USDA) monitors and enforces pesticide residues and tolerances in meat, milk, poultry, eggs and aquacultural foods.

The definition for food additive (FFDCA § 201(s)) excludes pesticide chemicals and pesticide chemical residues in or on a raw agricultural commodity or processed food and are therefore excluded from regulation under § 408 of FFDCA. However, tolerance and exemption from tolerance of pesticide chemicals and pesticide chemical residues in or on food products must be established by EPA under § 408 of the FFDCA and enforced by FDA. Such tolerances or exemptions are listed in Title 40 CFR part 180. Table 2 lists some examples of antimicrobials regulated by EPA, FDA, and EPA and FDA, respectively.

1.4. Chemical Oxidants

In all chemical processes, when one substance is oxidized another one must be reduced. Oxidation is an increase in the positive valance state of a substance, such as by the removal of one or more electrons from an atom or ion. Conversely, reduction is the addition of one or more electrons to a molecule, resulting in a less positive or more negative valance state. Ozone, hydrogen peroxide, hypochlorites, chlorine and chlorine dioxide are the oxidizing agents most commonly used for the chemical treatment of organic contaminants. Antimicrobial agents such as sodium hypochlorite and hydrogen peroxide work directly via radical-mediated reactions to oxidize organic material (Chapman, 2003). This section will focus on the chemical, physical and antimicrobial properties of sodium hypochlorite and hydrogen peroxide.

1.4.1. Sodium Hypochlorite. The majority of utilities in the United States have shifted from using gaseous chlorine to sodium hypochlorite, often referred to as liquid bleach or soda bleach liquor (Casson and Bess, 2003). As of today, sodium hypochlorite is the most widely used form of chlorinated bleach for laundry, household and general disinfection (White, 1999).

Table 2. Examples of antimicrobials regulated by FDA, EPA, and FDA and EPA (Modified from Knight and Cooke, 2002).

Antimicrobial Usage	Antimicrobial Regulation
Antimicrobials included in, or applied to, food packaging without regard to whether the substance is intended to have an ongoing effect on portion of the packaging (FFDCA §201(q)(1)(B)(ii); 21 U.S.C 321(q)(1)(B)(ii)).	FDA as food additives
Food-contact substances (other than components of food packaging) with no intended ongoing effect on any portion of the object (e.g., papermill slimicides, and antimicrobials used as preservatives in the production of rubber latices and water-based adhesives and coatings (FFDCA §201(q)(1)(B)(ii); 21 U.S.C 321(q)(1)(B)(ii)).	FDA
Antimicrobial substances incorporated into permanent or semi-permanent food contact articles, other than food packaging, with an ongoing antimicrobial effect on any part of the object except the food-contact surface (e.g., antimicrobials incorporated into cutting boards, coatings of conveyer belts, plastic tubing) (FFDCA §201(q)(1)(B)(ii); 21 U.S.C 321(q)(1)(B)(ii)).	FDA
Antimicrobials used on permanent and semi-permanent surfaces (e.g., a hard surface sanitizer for equipment that contact food) (FFDCA §201(q)(1)(B)(ii); 21 U.S.C 321(q)(1)(B)(ii)).	EPA Uses are subject to registration under FIFRA and to the tolerance or tolerance exemption requirements of FFDCA Section 408

Table 2 cont.

Antimicrobial Usage	Antimicrobial Regulation
Antimicrobials in treated articles marketed with claims that the antimicrobial is intended to have a pesticidal effect on the food contact surfaces (FFDCA §201(q)(1)(B)(ii); 21 U.S.C. 321 (q)(1)(B)(ii))	EPA Treated articles could be subject to the tolerance and tolerance exemption requirements of FFDCA Section 408
Antimicrobial and other pesticide products applied on raw agricultural commodities (RAC) and for which there is no processing in the field or in facilities receiving the RAC's (FFDCA § 201(q)(1)(B)(i); 21 U.S.C. 321(q)(1)(B)(i)	EPA Pesticides are subject to the tolerance and tolerance exemption requirements of FFDCA Section 408
Antimicrobial applied to water that contacts food in facilities where food processing takes place (FFDCA §201 (q)(1)(B)(i); 21 U.S.C. 321(q)(1)(B)(i)).	FDA as a food additive FFDCA § 409 and EPA as a pesticide under FIFRA
Antimicrobial preservative that is a component of an article with food contact, but in which the antimicrobial has no intended ongoing effect on the article on the article's food contact surface (e.g., a preservative of the plastic or latex incorporated into a piece of equipment used in a food processing facility) (FFDCA §201(q)(1)(B)(ii); 21 U.S.C. 321(q)(1)(B)(ii)).	FDA as a food additive (either direct or secondary direct) under FFDCA Section 409 and by EPA as a pesticide under FIFRA.

1.4.1.1. Manufacture and Chemical Characteristics. The manufacture of sodium hypochlorite is a simple procedure that involves the reaction of chlorine with caustic soda in a batch or continuous process (Gordon et. al. 1995). The reaction is as follows:

$$2\ NaOH + Cl_2 \rightarrow NaOCl + NaCl + H_2O + Heat$$

The strength of a soda bleach solution is often expressed in terms of its available chlorine content as "trade percent" or "percent by volume". However, a more accurate expression is the actual weight percent of sodium hypochlorite. The relationship between these values can be demonstrated as follows (White, 1999):

$$\text{trade percent (percent by volume)} = \frac{\text{g/L available } Cl_2}{10}$$

$$\text{weight percent available } Cl_2 = \frac{\text{trade percent}}{\text{specific gravity of solution}}$$

Other commonly used terms include free available chlorine and available chlorine. Free available chlorine (FAC) refers to the concentration of molecular chlorine (Cl_2), hypochlorous acid (HOCl), and hypochlorite ion (OCl^-) in water expressed as available chlorine (Haas, 1999). Available chlorine is used to express the amount of chlorine in chlorine gas and hypochlorite salts (Hass, 1999). Commercial grade sodium hypochlorite can be produced by manufacturers at concentrations as high as 16 % chlorine, with typical concentrations ranging between 5 and 15% chlorine (Casson and Bess, 2003; White, 1999). However, significant losses of chlorine in higher sodium hypochlorite solutions are of major concern in the industry.

The stability of a sodium hypochlorite solution is greatly affected by factors such as concentration, light, pH, temperature and heavy metals. An increased reduction in the strength of a sodium hypochlorite solution is often observed with increasing hypochlorite concentration, temperature and holding time (Casson and Bess, 2003).

Liquid bleach typically has a pH between 11 and 13. In basic solution, hypochlorite ion (OCl^-) decomposes to form chlorate ion (ClO_3^-) (a toxic byproduct). This is a second order process and involves the reaction of OCl^- with chlorite ion OCl_2^- (an intermediate ion). The reaction is as follows (Bolyard and Fair, 1992):

$$2OCl^- \rightarrow ClO_2^- + Cl^- \quad \text{(slow reaction)}$$
$$OCl^- + ClO_2^- \rightarrow ClO_3^- + Cl^- \quad \text{(fast reaction)}$$

Lowering the pH of liquid bleach will accelerate ClO_3^- formation and the pH of the OCl^- solution will continue to decrease over time. This is represented by the following equation (Gordon et. al. 1997):

$$2HOCl + OCl^- \rightarrow ClO_3^- + 2H^+ + 2Cl^-$$

Mixing of sodium hypochlorite with any acid will result in the release of chlorine gas. At pH 9 and higher, the HOCl is almost completely dissociated to OCl^- (a very poor disinfectant) as indicated below (White, 1999):

$$HOCl \leftrightarrow H^+ + OCl^-$$

Dissociation of HOCl takes place very rapidly so equilibrium between HOCl and OCl^- is always maintained, even though HOCl is continuously being used (Gordon et. al. 1997, White, 1999). Increasing the temperature of the reacting solution increases the rate of HOCl dissociation (Gordon et. al., 1997).

Cyanuric acid, commonly known as “conditioner” or “stabilizer”, has a pH of 4.0 and is added to chlorine containing compounds in outdoor pools to shield chlorine from the degrading effects of U/V light (Home Comcast, 2004). The stabilizer is added at levels between 30 ppm to 50 ppm with a maximum of 100 ppm and a lower limit of 10 ppm.

Hypochlorite decomposition may be catalyzed by transition metals. According to Gordon et. al. (1995), the maximum concentration of transition metal ions that does not catalyze bleach

decomposition is ~0.1 mg/L Ni^{2+} and ~1 mg/L Cu^{2+}. However, either ferric iron or manganese, behave as catalysts for sodium hypochlorite decomposition.

The most stable liquid bleach solutions are those of low sodium hypochlorite concentrations (10%), with iron, nickel and copper concentrations of less than 0.5 mg/L, stored in the absence of light, at a temperature of about 21˚C with a pH of 11 (White, 1999).

1.4.1.2. Antimicrobial Properties of Sodium Hypochlorite. The active agent of sodium hypochlorite is hypochlorous acid, which forms upon the hydrolysis of the hypochlorite ion. Hypochlorous acid is a weak acid but powerful oxidant. The reaction is as follows (White 1999):

$$NaOCl + H_2O \rightarrow HOCl + NaOH$$

Hypochlorous acid is both sporicidal and bactericidal and with the exception of chlorine dioxide, is the most effective biocide of the chlorine compounds (Russel, 1983). Hypochlorous acid has been used for the disinfection of drinking water since 1908 (Gordon et. al., 1993). The germicidal efficacy of HOCl is attributed to both its low molecular weight and its lack of electrical charge, which enables the molecule to penetrate cell walls with relative ease.

The activity of hypochlorous acid is a function of pH and temperature (White, 1999). The disinfecting efficiency of the chlorine residue decreases significantly as the pH rises. Reports by Khan and Kasha (1994) indicated that acidification of sodium hypochlorite yielded singlet oxygen (onset at pH 8, 1268 nm) before the appearance of chlorine (onset at pH 5.5). The highest emission intensity was observed by adding 200 µl concentrated HCl to a 5 ml 0.6 M sodium hypochlorite solution with a final pH of 5. Singlet oxygen is a powerful oxidant and as such a strong antimicrobial agent that reacts with lipids, proteins and nucleic acids.

1.4.2. Hydrogen Peroxide

1.4.2.1. Manufacture and Chemical Characteristics. Hydrogen peroxide is a clear and colorless liquid that is miscible with water and soluble in organic solvents at concentrations higher than 65% (Jones, 1999). Hydrogen peroxide is a relatively weak oxidizing agent. It can oxidize certain substrates such as olefins, aromatic hydrocarbons and alkanes (Jones, 1999). However, for most applications some form of activation is needed. A summary of the various forms of active oxidants derived from hydrogen peroxide is given in Figure 1. In alkaline environments, hydrogen peroxide dissociates into hydrogen and perhydroxyl ions (Jones, 1999). The reaction is as follows:

$$H_2O_2 \rightarrow H^+ + HOO^-$$

The perhydroxyl anion is a powerful nucleophile. It can also generate powerful oxidants when reacting with electron deficient acyl compounds or with nitriles (Perez-Benito, 2001). In strong acidic conditions, hydrogen peroxide can be protonated or converted to hydroxyl cation (Jones, 1999). The decomposition of hydrogen peroxide is presented below:

$$H_2O_2 + H^+ \rightarrow H_3O_2$$

$$H_2O_2 + HA \rightarrow H_3O_2^+ + A^-$$

Hydrogen peroxide decomposition can take place by the action of light (Fiorenza and Ward, 1997), transition metal ions (Halliwell and Gutteridge, 1985), and enzymes such as catalases and peroxidases (Adams et. al., 2002).

Hydrogen peroxide can be generated *in vivo* and industrially. *In vivo*, hydrogen peroxide is generated in small amounts by almost all microorganisms growing aerobically. Under aerobic conditions, oxygen is used as an electron acceptor with hydrogen peroxide or water as the reduced products. The cyanobacterium *Anacystis nidulans* generates H_2O_2 during

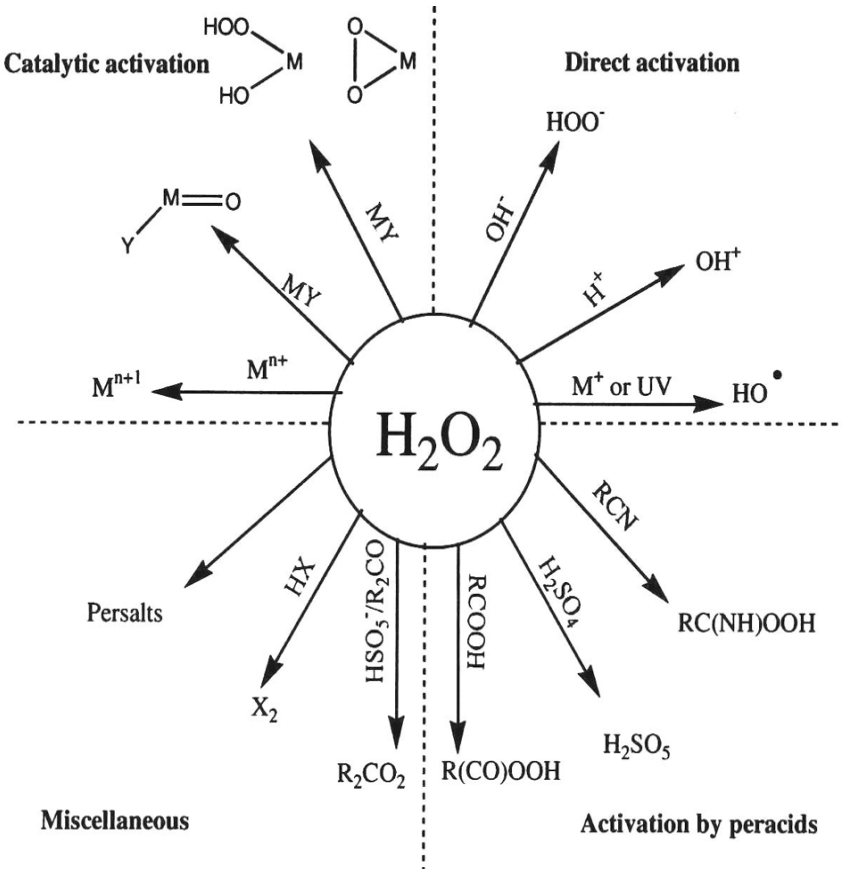


Figure 1. Activation of hydrogen peroxide (Jones, 1999).

photoautotrophic growth (Roncel et. al., 1989). *Streptococcus faecalis* oxidizes glycerol 3-phosphate with the release of H_2O_2 (Clarke and Knowles, 1980). *Streptococcus pneumoniae* generates hydrogen peroxide through the action of pyruvate oxidase under conditions of aerobic growth (Pericone,C. D. et. al., 2000). Species of the genus *propionibacterium* produce porphyrins that can lead to the formation of hydrogen peroxide and hydroxyl radicals, by porphyrin mediated photosensitizing reactions in the presence of light and oxygen (Juven and Pierson, 1996). Also, any superoxide generated enzymatically at physiological pH can produce hydrogen peroxide through the dismutation reaction either spontaneously or through metal and enzyme catalyzed reactions (DiGuiseppi and Fridovich, 1981, Frelon et. al., 2003). Industrial manufacture of hydrogen peroxide relies mostly on the anthraquinone- anthrahydroquinone process or the AO process, as it is commonly known (Jones, 1999).

1.4.2.2. Antimicrobial Properties. Hydrogen peroxide has both bactericidal and sporicidal properties. The antimicrobial properties of hydrogen peroxide have been long well documented and are influenced by a variety of factors such as pH, concentration, presence of trace metals, differences in bacterial strains and temperature (Russel, 1990 and Juven and Pierson, 1996)

In biological systems, hydrogen peroxide is a weak oxidant that may oxidize thiol groups in proteins (e.g., glyceraldehydes-3-phosphate-NAD+ oxidoreductase, the key enzyme in glycolysis) and polyunsaturated fatty acyl groups in lipids. Oxidative killing of metabolically active cells by hydrogen peroxide is apparently attributed to the generation of a reactive, cytotoxic and powerful oxidant, the hydroxyl radical (Shin et. al., 1994). Hydroxyl radicals are very small molecules and can easily diffuse through cell membranes damaging nucleic acids, proteins and lipids (Woznichak et. al., 2000, Juven an d Pierson, 1996). The half life of a hydroxyl radical is approximately 10^{-9} sec at 37˚C (Frei, 1994). Hydroxyl radical generation can

take place under the following conditions: (1) The non-enzymatic reaction of hydrogen peroxide with transition metals (Perez-Benito, 2001, De Laat and Gallard, 1999); (2) Superoxide anions interacting with hydrogen peroxide in the presence of Fe^{3+}, the Fenton mechanism or the iron catalyzed Haber-Weiss reaction (Halliwell and Gutteridge, 1985; DiGuiseppi and Fridovich, 1981; Halliwell and Gutteridge, 1992); the reaction is shown below:

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$
$$Fe^{2+} + H_2O_2 \rightarrow OH\bullet + OH^- Fe^{3+}$$

and (3) UV irradiation (Waites et. al., 1988). The reaction is as follows:

$$H_2O_2 \rightarrow OH\bullet + OH\bullet$$

Reports by Khan and Kasha (1994) have shown singlet oxygen as the highly reactive oxidant and hydroxyl radical as a short-lived transient intermediate in the Haber-Weiss reaction when mixing aqueous hydrogen peroxide with a solution of potassium superoxide. The superoxide anion is expected to be an efficient generator of singlet oxygen over a narrow concentration range. Shown below are three mechanisms proposed to explain the generation of singlet oxygen during the Haber-Weiss reaction. The reactions are as follow:

(1) Haber-Weiss: $O_2^- + H_2O_2 \rightarrow {}^1O_2 + OH\bullet + OH^-$

(2) Electron transfer: $OH\bullet + O_2^- \rightarrow {}^1O_2 + OH^-$

(3) Dismutation: $2H^+ + 2O_2^- \rightarrow {}^1O_2 + H_2O_2$

Increase microbicidal activity of hydrogen peroxide due to the enzyme peroxidase has been reported in the presence of halide ions (Klebanoff, 1968). Lactoperoxidase, found in milk, saliva and other biological fluids (Brul and Coote, 1999) and myeloperoxidase, found in neutrophiles and monocytes (Juven and Pierson, 1996) are among the peroxidases that can function in this way. The enzyme lactoperoxidase catalyses the oxidation of thiocyanate to

hypothiocyanite in the presence of hydrogen peroxide. Hypothiocyanite is a weak oxidizing agent and the major antimicrobial product of the system. The myeloperoxidase system requires a reaction between hydrogen peroxide and chloride ions, resulting in the formation of hypochlorous acid (Stanier, 1986, Panasenko, 1997).

1.5. Antimicrobial Applications of Sodium Hypochlorite and Hydrogen Peroxide

1.5.1. The Bacterial Cell

1.5.1.1. The Bacterial Cell and Antimicrobial Interaction. The bacterial cell provides three targets for biocides: the cell wall, cytoplasmic membrane and cytoplasm. However, it is possible that a biocide can interact with one or all three regions of the bacterial cell to have an antimicrobial effect. A summary of potential targets for biocide interaction within the bacterial cell is given in Figure 2.

The cell wall is comprised of an open network of peptidoglycan (with a lipopolysaccharide overlayer for Gram-negative bacteria) that serves as an excellent target for antimicrobials. The cell wall structure of Gram-negative bacteria and the presence of an outer envelope add to their resistance to antimicrobials when compared to their counterparts, the Gram-positive bacteria (Denyer and Maillard, 2002). The peptidoglycan layer and its associated anionic polymers provide access to the membrane of microorganisms to molecules with molecular weights ranging from 30 KDa to 57 KDa. Antimicrobials such as hydrogen peroxide, phenols, alcohols, aldehydes, QACs and biguanides are all small enough to cross the cell wall at ease (Lambert, 2002). Porins, which are large protein structures embedded within the outer membrane, not only allow the diffusion of cellular nutrients but can also serve as channels to hydrophilic biocides of molecular weight less than 600 Da (Denyer and Maillard, 2002).

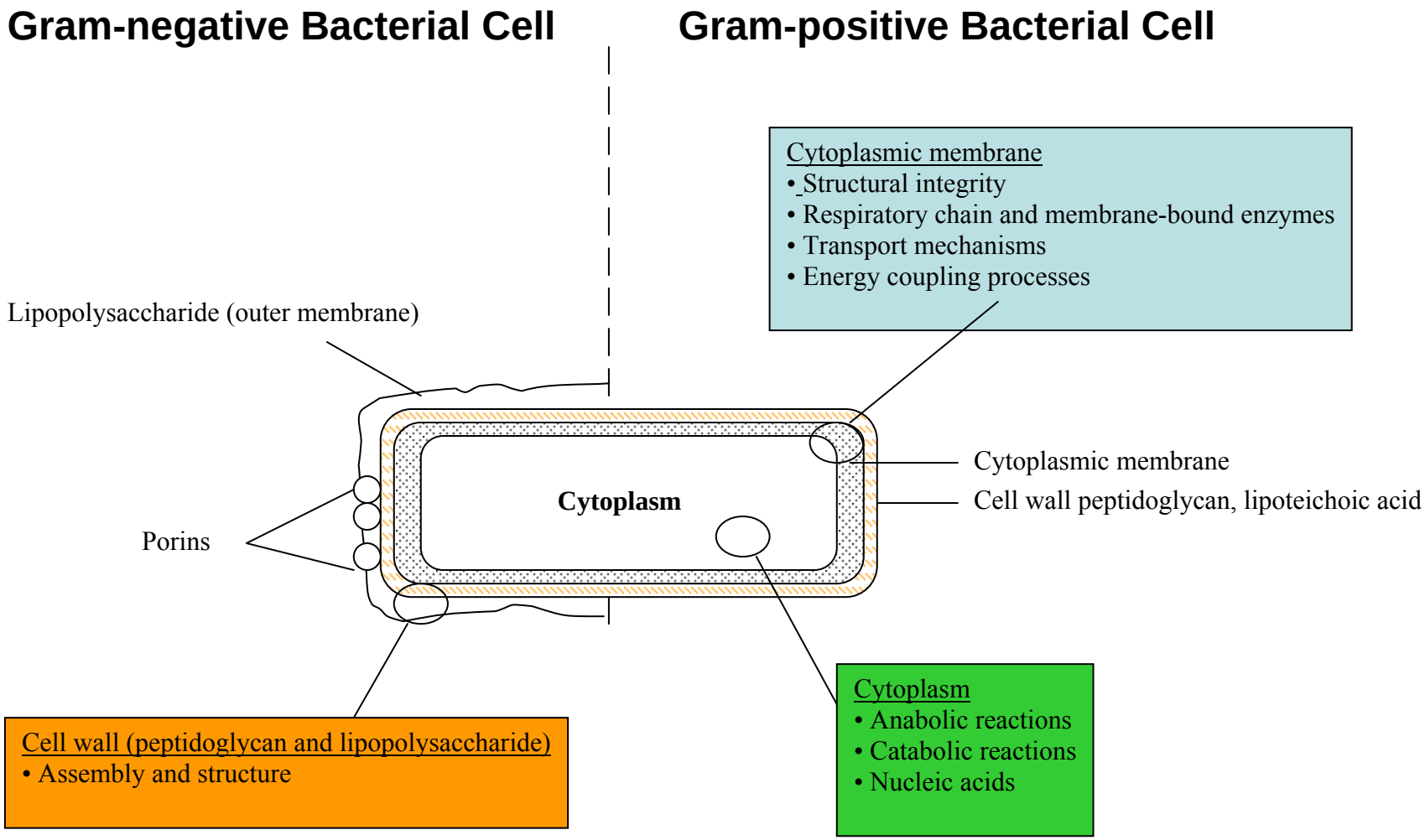


Figure 2. Potential biocide targets in bacterial cells (Modified from Denyer and Stewart, 1998).

The cytoplasmic membrane on the other hand, is a rich matrix of balanced interactions between phospholipid and enzymatic/structural proteins where intracellular homeostasis and transport/metabolisms are maintained, thus predisposing this region to biocide attack (Denyer and Stewart, 1998). The cytoplasm is by far the most common target for biocides due to the many catabolic and anabolic processes that take place here. Damage to the membrane can be attributed to one or more of the following events: (1) physical disruption (2) dissipation of the proton motive force and (3) inhibition of membrane associated enzyme activity (Maillard, 2002).

1.5.1.2. The Bacterial Cell and Hydrogen Peroxide or Sodium Hypochlorite Interaction. As mentioned previously, the generally proposed mechanism of action of H_2O_2 involves its breakdown to yield radicals such as the hydroxyl radical, a short lived free radical that damages nucleic acids, proteins and lipids (Brul and Coote, 1999). Hydrogen peroxide can directly inactivate enzymes such as gluceraldehyde-3-phosphate, usually by oxidation of thiol groups. Exposure to high levels of hydrogen peroxide can lead to ATP depletion by inhibition of glycolysis.

The ability of hydrogen peroxide to cross the cell membrane rapidly and with ease allows it to exert its damage on deeper molecules such as nucleic acids. Once inside the cell, hydrogen peroxide can react with trace metals (e.g., iron, copper) to form hydroxyl radicals which result in numerous lesions to DNA and nucleoproteins. Damage includes base lesions, single-strand breaks and DNA-protein crosslinks (Wallace, 2002). Most bacterial cells rely on the action of catalase to degrade hydrogen peroxide. The enzymatic action of catalase breaks down hydrogen peroxide to water and oxygen. Nevertheless, damage can still occur due to the high diffusion rate of H_2O_2 into the cell and the inability of catalase to protect individual cells, especially when hydrogen peroxide is present in high concentrations. However, in high-density populations,

catalase positive cells produce enough enzymes to protect individual cells against toxic levels of hydrogen peroxide (Brul and Coote, 1999). Hydrogen peroxide concentrations needed to exert bactericidal and sporicidal effects vary, and are attributed to differences in bacterial strains and other environmental factors such as pH and temperature (Juven and Pierson, 1996). At a concentration of 6% (w/vol), hydrogen peroxide becomes bactericidal, but only slowly sporicidal. However, at 25°C and levels of between 10 and 20% (w/v), the concentration exponent is about 1.5 (Russell, 1983). Ukuku et al. (2001) reported that dipping cantaloupes in solution containing 5% hydrogen peroxide or 1000 mg/L chlorine for 5 min, within 24 h of inoculation, caused a 2 log10 cfu/cm^2 reduction in *E. coli*. However, the efficacy was less when the interval between inoculation and treatment exceeded 24 h. Reports by Annous et al. (2001) have indicated that 5% hydrogen peroxide at 20°C, 5% hydrogen peroxide at 50°C and 200 ppm of chlorine (pH 6.4) in 20°C washing solutions did not reduce *E. coli* populations on inoculated apples or in the cider made from these apples, probably due to the inability of the biocide to kill or remove inaccessible cells present in the calyx and stem areas of the apples. Sagripanti et al. (1997) reported resistance of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Clostridium perfringes*, *Salmonella typhimurium*, *E. coli* O157:H7 to germicidal agents such as, glutaraldehyde, hydrogen peroxide, sodium hypochlorite, formaldehyde, peracetic acid, cupric ascorbate and phenol. Hydrogen peroxide resistance has also been reported in few strains of *Listeria monocytogenes* during egg white processing (Muriana, 1997). Reports by Fletcher and Csonka (1998) have indicated that the presence of 0.3 M NaCl in growth media increased the resistance of *Salmonella typhimirium* to thermal death at 50°C and to the oxidizing agent hydrogen peroxide. On the other hand, amino acids such as histidine has been found to greatly augment the toxicity of hydrogen peroxide in *E. coli* cells (Brandi et al., 1991).

Hypochlorous acid reacts with a wide variety of biological molecules including proteins, DNA, cholesterol, lipids, free thiols and sulfides (Panasenko 1999, Noguchi, et. al. 2002, Hawkins et. al. 2003, Bloomfield and Arthur, 1989). The bactericidal activity of hypochlorite has been attributed to the formation of secondary products (chloramines) as they react with sub-cellular compounds such as ammonia ions and organic amines (Miche and Balandreau, 2001). Chloramines are toxic compounds capable of diffusing through cell membranes and reaching intracellular components such as DNA. Chloramines disrupt hydrogen bonding resulting in the dissociation of DNA (Hawkins et. al. 2003). Studies have demonstrated that radicals that result from the decomposition of chloramines on hypochlorous acid treated proteins can cause damage to other substrates as well (Hawkins et. al. 2003). The oxidation of proteins by hypochlorous acid can lead to side chain modification. Many of these reactions occur primarily with thiols, sulfides, amines and to a lesser extent with amides and aromatic rings (Hawkins et. al. 2003). In addition, some chloramines (e.g., chloramines T) can react with hydrogen peroxide resulting in the generation of singlet oxygen (Khan and Kasha, 1994). The effectiveness of H_2O_2, like NaOCl, is dependent on concentration, pH, temperature, microbial strain, and the presence of organic materials.

1.5.2. The Bacterial Spore

1.5.2.1. The Bacterial Spore and Antimicrobial Interaction. Bacterial spores are highly resistant to antibacterial agents. The most important spore formers are members of the genera *Bacillus* and *Clostridium*; however, other bacteria such as *Sporosarcinae, Desulfomaculum*, *Sporolactobacillus*, and thermophilic actinomycetes also produce spores (Russell, 1990). The bacterial spore is a complex entity and its resistance appears to be associated with both the spore coats and the cortex. A schematic representation of a typical bacterial spore is depicted in Figure

3. The spore coat makes up a large portion of the spore and is mostly protein, whereas the cortex is composed of peptidoglycan. About 45 to 60% of the muramic acid residues within the peptidoglycan layer do not have either a peptide or an N-acetyl residue but instead a muramic lactam (Warth, 1978). The protoplast is the location of DNA, RNA, dipicolinic acid, and most of the calcium, manganese, potassium and phosphorous present in the spore. Sporicidal and sporostatic effects of antimicrobial agents are not limited to the spore coats, cortex or protoplast but may also be effective during germination and outgrowth processes.

1.5.2.2. The Bacterial Spore and Hydrogen Peroxide or Sodium Hypochlorite Interaction. Awareness of the sporicidal action of hydrogen peroxide and hypochlorite salts has led to their use in industry especially for aseptic processing and packaging. In the food industry, these agents are used independently or in combination with other compounds for the sterilization of packaging materials for aseptic filling (Toledo, 1975), in sanitizing of hatching eggs (Sheldon and Brake, 1991), low-temperature pasteurization of liquid whole eggs (Unluturk and Turantas, 1987), and as a decontaminant of broiler carcasses (Lillard and Thomson, 1983) and in milk processing plants (Giffel et al., 1996). Other uses include sterilization of heat sensitive materials such as endoscopes and kidney dialysis units (Palop et al., 1998). Experimental evidence indicates that either hydrogen peroxide or hypochlorite can cause disruption and extraction of spore coat material, facilitating disinfectant penetration to the cortex and protoplast (Russell, 1983). Palop et al. (1998) proposed that hydroperoxides have an effect on multiple enzymes within the spore and cumulative damage leads to loss of viability. Unlike with vegetative cells, killing of spores by hydrogen peroxide does not involve DNA damage. DNA in the dormant spore is protected by small, acid-soluble proteins of the α/β type (Bagyan et al., 1998). Spore coats of Bacillus sp. are generally not implicated in hydrogen peroxide resistance but are known

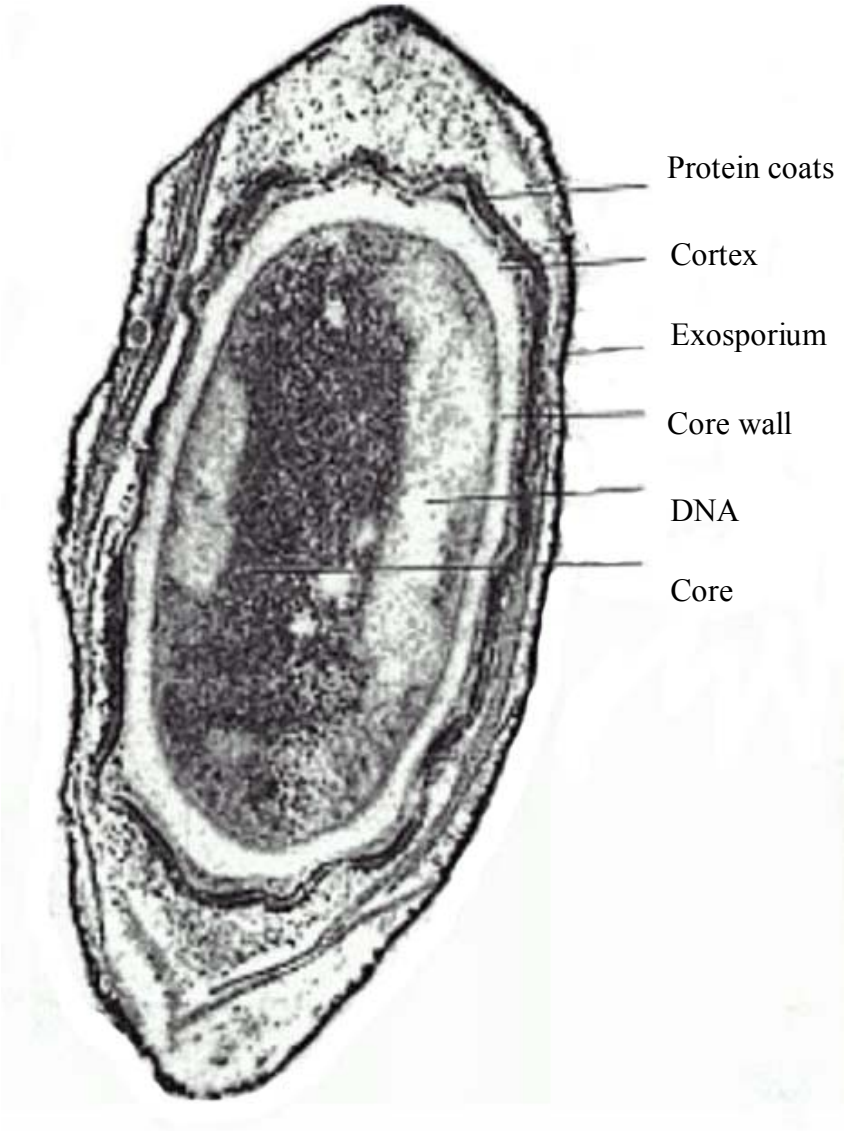


Figure 3. The bacterial spore (http://bioweb.usu.edu/takemoto/5300/Images.htm).

to be important in *Clostridium bifermentans* survival from hydrogen peroxide stress (Bayliss and Waites, 1976). Freshly prepared hypochlorite solutions, buffered to about pH 7.6, are rapidly sporicidal (Russell, 1990). Mixtures of 1.5 to 4% sodium hydroxide with sodium hypochlorite (200 ppm available chlorine) are much more sporicidal than each compound used alone (Russell, 1990). Bloomfield and Arthur (1992) reported that at 200 ppm available chlorine, sodium hypochlorite and sodium dichloroisocyanurate, in the presence of 0.4% sodium hydroxide, produced degradation of *Bacillus subtilis* spore coats and cortex facilitating diffusion to the protoplast. Damage of the protoplast membrane resulted in dipicolinic acid release and solubilization of hexosamines. The presence of sodium hydroxide allowed these formulations to be used at much lower concentrations. In addition, work by Foegeding and Busta (1983) showed that chlorine treatment of *Bacillus subtilis*, *Bacillus cereus*, *Clostridium bifermentans* or *Clostridium botulinum* spores increased their sensitivity to lysozyme. Orr and Beuchat (1999) obtained reductions of 2.2, 0.4 and 0.1 logs in the number of viable *Alicyclobacillus acidoterrestris* spores when they were suspended in 200 ppm chlorine, 500 ppm acidified sodium chlorite, or 0.2% hydrogen peroxide solutions for 10 min at 23°C. However, spore viability was reduced by more than 5 logs when exposed to either 1000 ppm chlorine or 4% hydrogen peroxide. Killing of *Bacillus sp*. spores by hydrogen peroxide, at elevated but sub-lethal temperatures and at neutral pH, without lysis have been reported (Shin et al., 1994). However, prolonged exposures to higher concentrations caused secondary lytic processes that damaged the coat, cortex and protoplast. Sagripanti and Bonifacino (1996) indicated that 10% hydrogen peroxide at room temperature is ineffective against *Bacillus subtilis* subsp. *globigii* and that a high concentration (35%) and temperature (80°C) are required for the destruction of spores. It was also concluded that hypochlorite at pH 11 was nearly ineffective at either 20 or 8°C and

showed a moderate spore inactivation at 40°C after 30 min. However, it was very active at neutral pH, but highly unstable.

1.5.3. Biofilms

1.5.3.1. Bacterial Biofilms and Antimicrobial Interaction. A mechanism of adaptive resistance to disinfectants is the formation of slime materials and/or biofilms (Heinzel, 1998). Biofilms are considered as the reversible and irreversible bacterial sorption and growth of cells at a surface (biotic or abiotic) with the production of extracellular polymeric substances (EPS) (Morton et al., 1998). Biofilms are not only highly complex but ubiquitous and can occur in both natural and man-made environments.

EPS is also called the glycocalyx and it is a hydrated polyanionic polysaccharide matrix produced by polymerases affixed to the lipopolysaccharide component of the cell wall. Other substances often found in the biofilm matrix include DNA, RNA, proteins and enzymes (Jass et. al., 2003). Growth within the biofilm matrix imparts protection to individual cells from often extreme environmental factors, including antimicrobial agents. In addition to protecting cells from the environment, nutrient acquisition, phenotypic variation and intercellular communication have been observed within biofilms (Jass et. al., 2003).

The increased antimicrobial resistance of microorganisms within biofilms is not entirely attributable to the failure of an agent to penetrate the full depth of the biofilm (Costerton et. al., 1999). Instead, a second hypothesis to explain biofilm resistance to antimicrobial agents has been proposed and it involves cells experiencing a nutrient limitation which causes them to enter a slow growing state where they do not readily take up antimicrobials (Cochran et al., 2000). A third mechanism of reduced biofilm susceptibility is that at least some of the cells in a biofilm adopt a distinct and relatively protected phenotype (Cochran et al., 2000). Experimental evidence

suggests that during biofilm formation, transcription of specific genes take place (Costerton et. al., 1999). Such observations have been reported with algC, algD, and algU genes from *Pseudomonas aeruginosa*, which are involved in the synthesis of the polysaccharide alginate (Costerton et. al., 1999). Cochran et al. (2000) has proposed that in addition to some genes becoming expressed during biofilm formation, some of the resulting gene products may reduce the susceptibility of the cell to antimicrobials. Biofilms can also serve as an entrapment for organic and inorganic materials. Beta-lactamases have been detected within the glycocalyx of *Pseudomonas aeruginosa*, thus giving the whole biofilm community the potential for increased B-lactam resistance (Morton et al., 1998). Increased accumulations of catalase have also been reported within the glycocalyx, thus allowing cells more resistance to hydrogen peroxide.

To kill and/or remove biofilms, the biocide must penetrate the EPA and gain access to the microbial cell. Oxidizing compounds like chlorine or peroxides have nonspecific mode of action and because of variation in the chemical composition of biofilms, these compounds are often preferred. However, they are rapidly consumed by reaction with organic material.

1.5.3.2. Bacterial Biofilms and Hydrogen Peroxide or Sodium Hypochlorite Interaction. Hypochlorites/ hypochlorous acid penetrate biofilms poorly due to the neutralization of the active chlorine as it reacts with organic materials on the surface of biofilms (Stewart *et. al.* 2001;Chen and Stewart 1996; Xu *et. al.* 1996). Stewart *et. al.* (2001) reported mean log reductions of 1.1 and 0.4 after 60 min treatment of six days old *Pseudomonas aeruginosa* and *Klebsiella pneumonaie* biofilms with 1000 mg L^{-1} chlorosulfamate and alkaline sodium hypochlorite, respectively. Similar penetration effects due to reaction-diffusion interactions have been observed for hydrogen peroxide in *Pseudomonas aeruginosa* biofilms (Stewart et. al. 2000)

Microorganisms in biofilms are more resistant to chemicals than their planktonic counterparts (Wirtanen et al., 2001; Lechevallier et al., 1988; Cochran et al., 2000). Reports by Sagripanti and Bonifacino (2000) demonstrated that *Pseudomonas aeruginosa* is on average 300 fold more resistant when present on surfaces than in suspension. Cochran et al. (2000) reported that disinfection rate coefficients for *Pseudomonas aeruginosa* averaged $0.551 mg^{-1}min^{-1}$ for monochloramine and $3.1x10^{-4}$ $mg^{-1}min^{-1}$ for hydrogen peroxide; compared to 24 h biofilm disinfection rate coefficients of 0.291 $mg^{-1}min^{-1}$ and $9.2x10^{-5}$ $mg^{-1}min^{-1}$ for monochloramine and hydrogen peroxide, respectively. Oxidizing formulations based on hydrogen peroxide (1.5% hydrogen peroxide plus 5-15% peracetic and acetic acid; 50% hydrogen peroxide plus 0.05% silver ions) have been reported to be effective against Gram-negative bacteria and biofilm constructs of *Bacillus subtilis* (Wirtanen et. al., 2001). Eginton et. al. (1998) reported that biofilms from *Pseudomonas aeruginosa* and *Staphylococcus epidermis* were less susceptible than their planktonic cells when exposed to sodium hypochlorite (0.02% or 0.015% w/v), Dodigen^{TM} (0.0015% w/v or 0.0006% w/v), sodium dodecylsulphate (6% w/v or 0.1% w/v) or tween-80 (6% w/v) for 5 min at 20˚C. Nevertheless, their attachment to surfaces was loosened by such treatments, with the exception of tween-80 treatment, which strengthened the attachment of *Staphylococcus epidermis* to stainless steel.

Despite the reduced activity of oxidizing compounds in the presence of organic matter, chlorine-based compounds are often the agents of choice for the removal of biofilms in food processing environments. The choice is based on their activity, availability and low cost.

1.6. Hydrogen Peroxide and Sodium Hypochlorite Reaction

1.6.1. Chemical Properties. The formation of singlet oxygen was first observed in 1963 by Khan and Kasha when carrying out a chemiluminescence experiment involving the decomposition of hydrogen peroxide in the presence of sodium hypochlorite (Khan and Kasha, 1963). The stoichiometry of the reaction is as follows:

$$NaClO + H_2O_2 \rightarrow NaCl + H_2O + O_2$$

Two emission bands were observed centered at 6334 Å (15788 cm^{-1}) and 7032 Å (14221 cm^{-1}). Khan and Kasha assumed that since the spacing between the bands (1567 cm-1) resembled the spacing between the levels of ground state molecular oxygen (1556 cm-1) the emission had to be that of electronically excited oxygen molecules. Their assumption was right but they incorrectly assigned the 6334 Å band to a solvent induced displacement of the origin of the 7032 Å transition observed with gaseous oxygen (Kearns, 1971). Later, Arnolg, Ogryzlo and Witzke correctly assigned the emission band to a transition involving a pair of singlet oxygen molecules, both in their singlet state (Kearns, 1971). Additional reports by Storch et. al. (1983) documented the generation of singlet oxygen upon mixing gaseous chlorine with concentrated basic hydrogen peroxide. Nevertheless, it was reported that the percent of singlet oxygen generation and the efficiency of chlorine gas utilization were dependent on the pH of the reaction mixture (Meyers et. al., 1980).

Later reports by Shiozawa (2000) indicated that other reactive oxygen species in addition to singlet oxygen resulted from the above chemical reaction. He confirmed the generation of hydroxyl radicals and superoxide anion radicals ($O_2^{-\bullet}$). It was also concluded that chlorine radicals ($Cl^{\bullet}$) existed on 5, 5, dimethyl-1-pyrroline-N-oxide (DMPO) spin adducts in the reaction

mixture. Figure 4 depicts the speculated mechanisms for $O_2^{-\bullet}$ and $OH^{\bullet}$ generation from sodium hypochlorite and hydrogen peroxide reactions.

Although the above reaction has been known for some time, considerable work remains to understand the complexity of this reaction.

1.6.2. Chemical and Physical Properties of Singlet Oxygen. Molecular oxygen exists as triplet and singlet oxygen. Stable ground state oxygen or triplet oxygen ($^3\Sigma^-_g$) is characterized by unpaired electrons with parallel spins in two different molecular orbitals. It behaves like a radical and is paramagnetic (Hudlicky, 1990). Excited state oxygen or singlet oxygen exists as $^1\Delta_g$ and $^1\Sigma^+_g$. Singlet oxygen ($^1\Delta_g$) is the lower energy species that reacts chemically as $^1O_2$ (Grossweiner, 2004). It has two unpaired electrons with antiparallel spins within the same orbital. Its excitation energy is 22.5 kcal/mol and it has a radiative decay lifetime of 45 min at very low gas pressures (Grossweiner, 2004). However, collisions with other molecules such as oxygen gas at 760 torr generate a lifetime of 14 min (Grossweiner, 2004). The higher energy state is $^1\Sigma^+_g$. In this state, two unpaired electrons occupy two different orbitals. This state decomposes to $^1\Delta_g$ so rapidly that it has no time to react with nearby molecules (Thiele and Elsner, 2001). The excitation energy is 37.5 Kcal/mole and the decay lifetime is 7 sec (Grossweiner, 2004). Occupancy of molecular orbitals of oxygen in its triplet ground state and its two excited singlet states is presented in Table 3.

Singlet oxygen can be generated by either photochemical or chemical methods. Photochemically, photosensitizing molecules (e.g., porphyrins, flavins, certain quinones) adsorb light and transfer the energy to triplet ground state molecular oxygen (found in micromolar concentrations in biological systems), thus elevating it into the excited singlet state (Phillips, 1994). Singlet oxygen can also be generated from a gaseous discharge by passing an electric

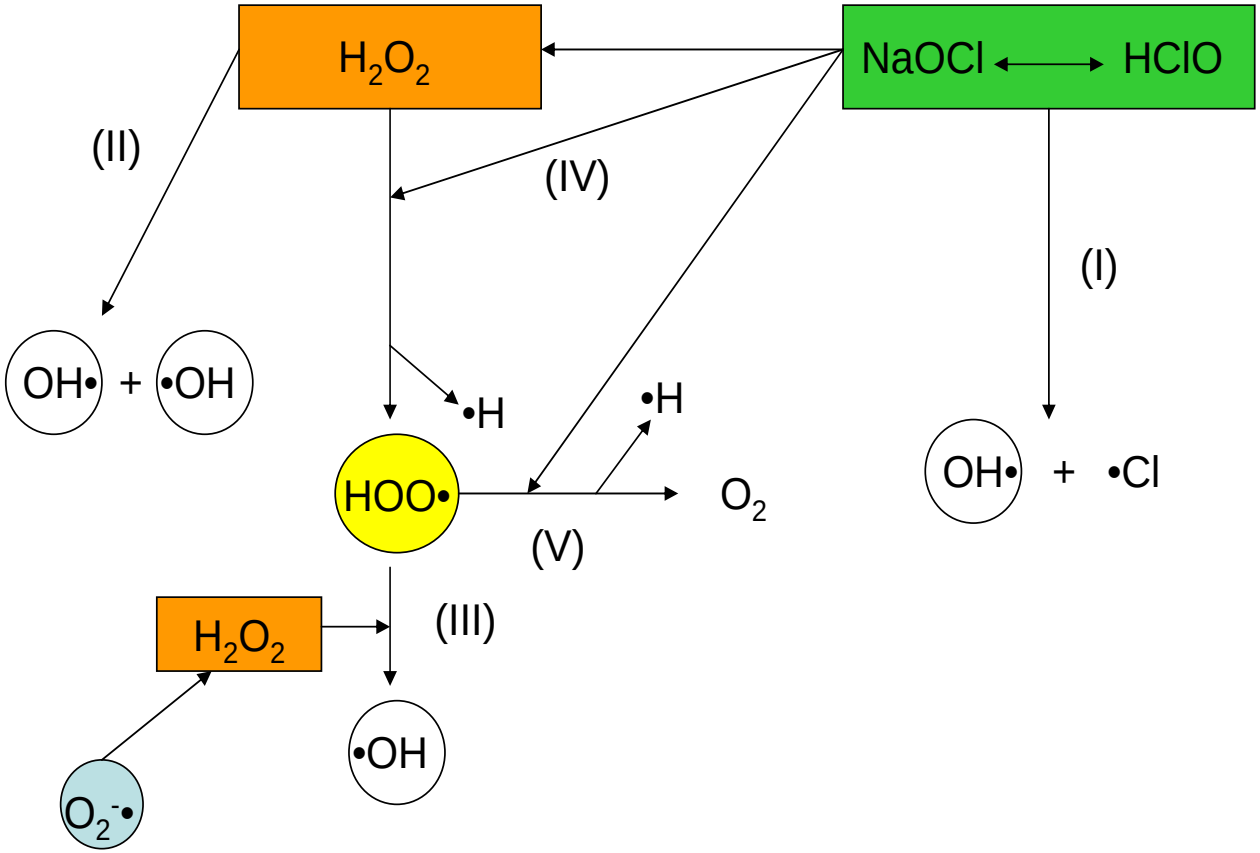


Figure 4. Speculated scheme for the generation of reactive oxygen species from the sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) reaction mixture. (I) HClO (NaClO) is decomposed to hydroxyl radical (OH•) and chlorine radical (•Cl) or chlorine ion (Cl^-). (II) H_2O_2 is decomposed to OH• + OH• by HOCl. (III) Generated superoxide radical (O_2^-•) acts on H_2O_2 and OH• is generated by the Haber-Weiss reaction. (IV) HClO extracts hydrogen ion from H_2O_2 and then hydroperoxyl radical (HOO•) and O_2^-• are generated. (V) H_2O_2 is decomposed by NaOCl, oxygen (O_2) is generated (effervescent reaction) (Shiozawa, 2000).

Table 3. Electron spins of molecular oxygen in its triplet and its two excited singlet states (Thiele and Elsner, 2001, Ameta et. al., 1990; Grossweiner, 2004).

States of the oxygen molecules	Occupancy of highest orbitals		Energy above ground state
Singlet oxygen state $^1O_2$ ($^1\Sigma_g^+$)	↑	↓	37.5 kcal/ mol
Singlet oxygen state $^1O_2$ ($^1\Delta_g$)	↑↓		22.5 kcal/ mol
Ground state $^3O_2$ ($^3\Sigma_g^-$)	↑	↑	

current through gaseous oxygen as well as oxygen atoms and ozone (Grossweiner, 2004). Chemical reactions involving myeloperoxides, lipid peroxidation, and hydroperoxides with peroxynitrite or hydrogen peroxide with hypohalites can generate singlet oxygen (Thiele and Elsner, 2001). The oldest method is the reaction of sodium hypochlorite with hydrogen peroxide. In addition, singlet oxygen plays an important role in biological reactions catalyzed by prostaglandin hydroperoxidase (Cadenas et. al., 1984) or the cytochrome P450 complex (Osada et. al., 1999).

The lifetime of singlet oxygen as well as that of radicals can be decreased by the addition of quenchers (e.g., carotenoids) or scavengers (tocopherols, histidine, and furan derivatives) (Karlsson, 1997). Activation or deactivation of singlet oxygen is also dependent on the type of solvent. Solvents with high vibrational frequencies provide the lowest stability. The lifetime of singlet oxygen can be in the range of 4 μs in water (highest frequency vibrations) and up to >1 ms in solvents lacking hydrogen and deuterium atoms (lowest frequency vibrations) (Thiele and Elsner, 2001 and Kearns, 1971). Nevertheless, the lifetime of singlet oxygen in these solvents may be compromised by the presence of traces of impurities.

1.6.3. Antimicrobial Properties of Singlet Oxygen and Reactive Oxygen Species (ROS). Singlet oxygen and ROS react with organic biomolecules (e.g., carbohydrates, proteins, lipids, nucleic acids) resulting in the modification of both their chemical structures and chemical properties. Singlet oxygen reacts directly with moieties of high electron density such as carbon double bonds which are found in most biomolecules, leading to protein oxidation, non-enzymatic lipid peroxidation and nucleic acid (RNA and DNA) damage. Singlet oxygen reacts with the s-*cis* conformations of 1, 3-dienes to form endoperoxides (Clennan, 1991). Other reactions involve the oxidation of olefins with allylic hydrogens to allylic hydroperoxides (Frimer, 1979) and 2,2 cycloadditions of singlet oxygen with electron rich substrates (Baumstark, 1985). The resulting hydroperoxides then cleave to initiate radical chain reactions. Decomposition of hydroperoxides, favored at high temperatures and by the presence of trace metals, leads to the formation of ROS such as alkoxy, peroxyl, superoxide anion and hydroxyl radicals. The highly reactive radicals can further propagate non-enzymatic lipid peroxidation by abstraction of a hydrogen molecule from unsaturated lipids. The chain cleavage products are in part comprised of reactive aldehydes which are known to electrophilically attack the thiols and amino moieties of proteins (Esterbauer et al., 1991). In the case of phospholipids, cleavage reaction fragments arise from the acyl chains, alkanes and fragments remaining bound to the phospholipids. These decomposition products can in turn react with proteins, DNA and thiol compounds. Lipid hydroperoxides may react with proteins by addition of the peroxyl radical to free amino groups (Kanner and Harel, 1985). Singlet oxygen and generated ROS, particularly hydroxyl radicals, can directly react with all four DNA bases resulting in the formation of a large number of characteristic products such as 8-hydroxyguanine, 8-hydroxyadenine, 2-hydroxyadenine and thymine glycols (Wallace, 2002).

The modified bases can result in single and double strand breaks, thus interfering with DNA replication.

Overall, direct and indirect (by the release of reactive compounds) damage to biological systems by singlet oxygen and ROS can result in the loss of cell membrane fluidity, leakage of cell enzymes and enzyme inactivation, disruption of cell homeostasis and interference with RNA, DNA and protein synthesis. Ultimately, the accumulation of damage as a result of oxidative stress will lead to cell death. Radicals derived primarily from singlet oxygen have also been implicated in the development of a wide variety of human diseases, including atherosclerosis (Jackson et. al., 1993), cancer (Cerutti and Trump, 1991), neurological disorders (Davidson et. al. 1988, Sokol, 1989) and aging (Taylor et. al., 1986).

1.7. Goal of This Study

The discovery of a stable, ROS and singlet oxygen generating system from a specific combination of hypochlorite and hydrogen peroxide (patent pending) led to the investigation into the utility of this "complex" as a broad spectrum biocide.

2. MATERIALS AND METHODS

2.1. Organisms and Maintenance

Salmonella typhimurium ATCC 14028, *Escherichia coli B* ATCC 23226 and *Pseudomonas aeruginosa* ATCC 19142 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained on nutrient agar (Difco, Detroit, MI) slants. *Xanthomonas maltophilia* strain #948 was a gift from Dr. Mark Ott (NASA, Houston, TX) and was maintained on nutrient agar slants supplemented with 0.2% yeast extract (YE) (Difco, Sparks, MD). *Leuconostoc mesenteroides* B512F, from the culture collection of Dr. Donal F. Day from the Audubon Sugar Institute at Louisiana State University (Baton Rouge, LA), was grown on Lactobacilli MRS (Difco, Sparks, MD) agar slants. *Bacillus subtilis, Bacillus pumilus* RJ 0055 and *Bacillus cereus* were obtained from the Department of Life Sciences at Louisiana State University. *Bacillus anthracis* (a sterne strain) was obtained from the Veterinary School at Louisiana State University. *Bacillus subtilis* ATCC 19659 spores were purchased from Presque Isle Cultures (Erie, PA). *Listeria monocytogenes* was obtained from Dr. Marlene Janes from the Department of Food Science at Louisiana State University. All *Bacillus spp*. vegetative cells and *Listeria monocytogenes* were maintained on Tryptic Soy Agar (TSA) slants (Difco, Detroit, MI). *Campylobacter jejuni* subsp. *Jejuni* ATCC 33560 was obtained from the American Type Culture Collection and grown on Brucella agar (BBL, Cockeysville, MD) slants supplemented with Campylobacter growth supplement (Oxoid, England) under microaerophilic conditions. The microaerophilic conditions were achieved using a GasPak™ anaerobic jar containing a single GasPak™ anaerobic system envelope. *Vibrio cholera* and *Vibrio parahaemolyticus* were wild isolates obtained from the Department of Food Science at Louisiana State University. They were stored on Marine agar (Difco, Sparks, MD) slants.

2.2. Preparation and Maintenance of Bacterial Spore Suspensions

Bacillus sp. were grown on a sporulation media containing 10g/L beef extract plus 2g/L yeast extract and 15g/L agar at 37ºC for 7 days. Cells were harvested, washed three times with distilled water and re-suspended in 5ml distilled water. Suspensions were heated at 75-80ºC for 20 min and cooled immediately on ice to inactivate vegetative cells. Spores were stored as a dense suspension in distilled water, at 4ºC, until needed. Spore verification was achieved by the Shaeffer-Fulton method (Schaeffer and Fulton, 1933) and plate counts on TSA were performed to determine the number of viable spores present in the suspension.

2.3. Biocide Treatment of Broth Cultures

2.3.1. Stock Solutions. Hydrogen peroxide (30%) (Sigma-Aldrich, St. Louis, MO), sodium hypochlorite (6%) (The Chlorox Co., Oakland, CA) or sodium hypochlorite (10-13%) (Sigma-Aldrich, Allentown, PA) were used as stocks. Solutions from stocks were prepared daily before each experiment by dilution of the concentrated materials with sterile-distilled water. For all the experiments described below, biocidal complex solutions were prepared by addition of hydrogen peroxide to sodium hypochlorite.

2.3.2. Determination of Minimum Inhibitory Concentration (MIC). The MIC of each component or combination thereof was obtained by mixing each component or combination in 15 ml test tubes and gradually adding the proper growth media of the organism being tested to bring it to the desired biocide concentration in a final volume of 10 ml. Each test tube was then inoculated with a 0.1 ml aliquot of the organism to achieve a final concentration of approximately 10^6 CFU ml^{-1}. The cultures were incubated for 24 hours at optimum growth temperatures (Table 4). The lowest concentration of biocide where there was no growth after 24 hours was taken as the MIC (Lorian, 1991).

2.3.3. Determination of Fractional Inhibitory Concentration (FIC). The FIC was calculated from the MIC values as described by the method of Berenbaum (Berenbaum, 1978). See equation below:

$$A_c/A_e + B_c/B_e$$

Where, A_c and B_c corresponds to the MIC values of biocides used in combination; and A_e and B_e corresponds to the MIC values of biocides used alone. When the sum of these fractions is 1, the combination is additive; <1, the combination is synergistic; and >1, the combination is antagonistic (Berenbaum, 1978). Table 4 lists the organisms, growth media and growth temperatures used for the MIC and FIC experiments.

Table 4. Organisms, growth media and growth temperatures used for the MIC and FIC experiments.

Organism	Media	Temperature (˚C)
S. typhimurium ATCC 14028	Nutrient broth	37
E. coli B ATCC 23226	Nutrient broth	35
P. aeruginosa ATCC 19142	Nutrient broth	35
X. maltophilia strain #948	Nutrient broth plus 0.2% YE	35
L. mesenteroides B512F	Lactobacilli MRS broth	30
B. subtilis	Tryptic soy broth	37
B. pumilus RJ 0055	Tryptic soy broth	37
B. cereus	Tryptic soy broth	37
B. anthracis	Tryptic soy broth	37
L. monocytogenes	Tryptic soy broth	35
C. jejuni subsp. *Jejuni* ATCC 33560	Brucella broth*	37
V. cholera	Marine broth 2216	30
V. parahaemolyticus	Marine broth 2216	30

*: Plus Campylobacter supplement.

2.4. Biocidal Complex Treatment of Distilled Water Cultures

2.4.1. MIC and FIC Determination of *B. subtilis* Spores. MIC and FIC values for sodium hypochlorite and hydrogen peroxide solutions against *B. subtilis* spores were determined as described previously. However, each component or combination thereof was obtained by mixing each component or combination in 15 ml test tubes and gradually adding distilled water instead of growth media to bring it to the desired biocide concentration in a final volume of 10 ml. *Bacillus subtilis* spores were added to a final concentration of approximately10^6 spores ml^{-1}. The activity of each biocide component was stopped at 5 min by mixing 0.1 ml and 1 ml aliquots with 5-10 ml D/E neutralizing broth. Samples were serially diluted, plated using the pour plate method in TSA and incubated at 37˚C for 24-48 h.

2.4.2. Ratio Effect on Biocidal Complex Activity. Different ratio combinations of hydrogen peroxide: sodium hypochlorite were tested for synergism against *B. subtilis* spores. The ratios tested were 10:1 (40,000 ppm: 4,000 ppm), 5:1 (50, 000 ppm: 10,000 ppm), 1:1 (40, 000 ppm: 40, 000 ppm), 1:5 (10,000 ppm: 50,000 ppm) , 1:10 (4,000 ppm: 40,000 ppm), 1:20 (2,000 ppm: 40,000 ppm), 1:50 (800 ppm: 40,000 ppm), and 1:100 (400 ppm: 40,000 ppm), respectively. Biocidal complex solutions were prepared in 15 ml test tubes and water was added to achieve the desired biocide concentration in a final volume of 10 ml. A 0.1 ml suspension of *B. subtilis* spores were added to a final concentration of approximately 10^6 spores ml^{-1}. The activity of the biocidal complex was stopped at 5 min by removing 0.1 ml and 1 ml aliquots and neutralizing them in 5-10 ml D/E neutralizing broth for at least 10 min. Proper dilutions were made and plated in duplicates by the pour plate method on TSA. Plates were incubated at 37˚C for 24-48 h.

2.5. Synergy of Sodium Peroxide with Sodium Hypochlorite

2.5.1. Preparation of Stock Solutions. Hydrogen peroxide (2.5% and 0.5%), sodium hypochlorite (5% and 0.5%) and sodium peroxide (0.5%) (Sigma-Aldrich, St. Louis, MO) solutions were prepared freshly before each experiment by dilution of concentrated stocks with sterile distilled water.

2.5.2. MIC and FIC Determination. MIC and FIC values were determined against *Pseudomonas aeruginosa* ATCC 19142 cells. Each biocide or combination was obtained by mixing each biocide component or combination in 15 ml test tubes and gradually adding nutrient broth to the desired biocide concentration and a final volume of 10 ml. Each test tube was then inoculated with 0.1 ml aliquot of an overnight culture of *P. aeruginosa* to a final concentration of approximately 10^6 ml^{-1}. The cultures were incubated for 24 hours at 35˚C. The lowest concentration of biocide where there was no growth after 24 hours was taken as the MIC (Lorian, 1991).

2.6. Antimicrobial Activity of Biocidal Complex against Spores on Stainless Steel Plates

2.6.1. Preparation of Biocidal Complex Solutions. Solution mixtures of 2,500 ppm hydrogen peroxide: 25,000 ppm sodium hypochlorite or 25,000 ppm sodium hypochlorite were prepared from 30% hydrogen peroxide and 6% sodium hypochlorite. All solutions were prepared with sterile nanopure water. Approximately a 100 ml solution each of biocidal complex and sodium hypochlorite were prepared and stored for two days at 4˚C, 24˚C and 37˚C. All solutions were allowed to adjust to room temperature by setting them aside for 20 min prior to testing.

2.6.2. Preparation of Stainless Steel Plates. Stainless steel plates (Type 316) measuring 1cm x 1cm were used in this study. Plates were immersed for 24 h in 1 N NaOH to remove any surface residue, rinsed with distilled water twice and then sterilized prior to use, by autoclaving at 121˚C

for 15 min. Spore suspensions (1 ml) of *Bacillus subtilis* (10^7 spores ml^{-1}) were added onto each plate and allowed to dry for 2-3 h in an incubator at 75˚C.

2.6.3. Effect of Time of Exposure and Temperature on Biocide Activity. Stainless steel plates with approximately 10^6 *B. subtilis* spores cm^{-2} on the surface were submerged in 0.5 ml of each test solution or distilled water for 1 min, 5 min, 15 min and 30 min contact times. Immediately after exposure to each test solution, each plate was transferred to a test tube containing 5 ml D/E neutralizing broth (Difco, Sparks, MD) and 0.5 g of glass beads (3 mm) (Fisher Scientific, Fair Lawn, NJ). All samples were vortex-mixed for 1 min, diluted in phosphate buffered solution (68g KH_2PO_4 / L, pH 7.0) or in distilled water and plated in duplicate on TSA plates. The TSA plates were incubated for 24-48 hours at 35˚C. All experiments were performed in duplicate.

2.6.4. Effect of pH on Biocide Activity. The pH of the test biocide solution was adjusted to 6.5 with concentrated HCl. Its biocide activity was tested against *B. subtilis* spore-contaminated stainless steel plates with a 15 min exposure at 4˚C. The testing procedure described above was followed.

2.7. Biocide Stability

2.7.1. Preparation and Storage of Biocidal Complex Solutions. Volumes (100 ml) of hydrogen peroxide and sodium hypochlorite mixtures at a 1:10 ratio (2,500 ppm: 25,000 ppm, respectively) and controls were prepared from 30% and 6% stocks, respectively. Solutions at pH 11 and adjusted pH of 6.5 were incubated at 4˚C, 24˚C and 37˚C for 42 days in the presence or absence of U/V light. All solutions were prepared with sterile distilled water and allowed to adjust to room temperature for 20 min prior to testing.

2.7.2. Effect of Storage on Biocidal Complex Activity. Stainless steel plates with approximately 10^6 *B. subtilis* spores cm^{-2} on the surface were submerged in 0.5 ml of each test solution (pH 11) or distilled water for a15 min contact time. Immediately after exposure to each test solution , each plate was transferred to a test tube containing 5 ml D/E neutralizing broth (Difco, Sparks, MD) and 0.5 g of glass beads (3 mm) (Fisher Scientific, Fair Lawn, NJ). All samples were vortex-mixed for 1 min, diluted in phosphate buffered solution (68g KH_2PO_4 / L, pH 7.0) or in distilled water and plated in duplicate on TSA plates. The above procedure was repeated on day 2, 4, 7 and 42. The TSA plates were incubated for 24-48 hours at 35˚C. All experiments were performed in duplicate.

2.7.3. Effect of Temperature, pH and U/V Light. The activity of test solutions with pH 11 or adjusted pH of 6.5 stored at 4˚C, 24˚C or 37˚C in the presence or absence of U/V light was randomly monitored using UV/Visible spectrophotometry.

2.7.4. Spectrophotometer Readings. Differential scans from 200 nm to 450 nm of the hydrogen peroxide and sodium hypochlorite mixture and each biocide component against distilled water were mode randomly for up to 42 days. Approximately 0.1 ml of sample was withdrawn, placed in a quartz cuvette with 1 cm pathlength containing 1.9 ml distilled water and the absorbance collected using a DU 800 UV/Visible spectrophotometer (Beckman Coulter Inc., Fullerton, CA).

2.7.5. Half-life Calculation. The peak area of each scan collected (with a maximum peak at 300 nm) was calculated using MatLab Student V 5.3 (MathWorks, Inc, Natick, MA). The area of each sample (Y ordinate) was plotted against time (X ordinate) and the equation of the line calculated. The half life of each sample was determined by entering a value of Y=0 and dividing time in half.

2.8. Singlet Oxygen Detection

2.8.1. Fluorescence Emission Spectra. Trans-1 (2'-methoxyvinyl) pyrine (t-MVP) was purchased from Molecular Probes, Inc. (Eugene, OR) and used to detect the presence of singlet oxygen. Reaction of singlet oxygen with t-MVP yields a dioxetane intermediate that generates chemiluminescence upon decomposition to 1-pyrenecarboxaldehyde (Figure 5). The emission spectra was determined using a 0.22 m Spex Fluorolo 6 1680 double spectrometer with excitation and emission wavelengths of 366 nm and 381-600 nm, respectively.

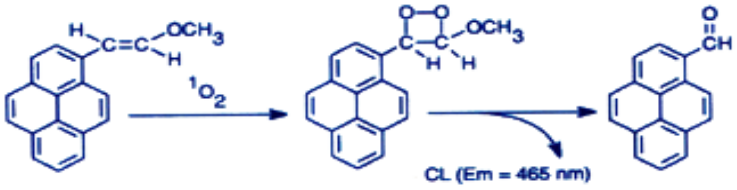


Figure 5. Reaction of trans-1- (2'-methoxyvinyl) pyrene with singlet oxygen.

2.8.2. Sample Preparation. Approximately 100 nmol of t-MVP were mixed with 0.3 ml of a 1:10 solution containing 2,500 ppm hydrogen peroxide: 25,000 ppm sodium hypochlorite solution and made up to a final volume of 3 ml with 0.1 M sodium dodecyl sulfate (Matheson Coleman & Bell Manufacturing Chemists, Norwood, OH) .One ml of this suspension was withdrawn, placed in a 1.5 ml quartz cuvette and the emission spectra determined. The above procedure was repeated, triggering the reaction with 10 µl of a suspension of *B. cereus* spores to a final concentration of 10^7spores ml^{-1}.

2.9. Response of *Pseudomonas aeruginosa* to Biocide

2.9.1. Bacterial Culture. *Pseudomonas aeruginosa* ATCC 19142 cultures were grown on Pseudomonas P agar and stored at 4˚C until used.

2.9.2. Substrata Preparation. Stainless steel (Type 304) and aluminum (Alloy 3003) plates measuring 1 in wide by 2.5 in long by 3mm deep were sand blasted to remove any scratches on the surface. Before each experiment, the stainless steel plates were cleaned by dipping in acetone, air dried, rinsed in nanopure water, soaked in 2 N HCl for 2 h, rinsed once with deionized water and then with nanopure water and allowed to air dry (Stewart, 2001). Aluminum plates were dipped in acetone, air dried and rinsed at least three times with nanopure water. Plates were then autoclaved for 15 min at 121˚C.

2.9.3. Preparation of Biocidal Complex Solutions. A hydrogen peroxide and sodium hypochlorite complex solution (2,500 ppm: 25,000 ppm, respectively) was prepared from stocks of 30% hydrogen peroxide and 10% sodium hypochlorite.

2.9.4. Biofilm System. An overnight culture of *Pseudomonas aeruginosa* was grown in TSB at 35˚C using a shaker-incubator at 150 rpm. A 5 ml inoculum of the overnight culture was inoculated into two separate 2 L beakers each containing 1200 ml TSB and vertically suspended aluminum or stainless steel plates (Figure 6). The growth in the medium was monitored by absorbance at 660 nm and kept below 0.4 by addition of fresh medium. This was achieved by replacing 800 ml of the culture broth with the same volume of fresh medium every two days, for a total of six days.

2.9.5. Biofilm Disinfection. Test coupons were harvested on day six when they reached approximately 8-9 log in^{-2} surface cells. Each test surface was rinsed with 10 ml distilled water and suspended in 30 ml of the biocide complex at a concentration of 2,500 ppm hydrogen

Figure 6. Growth of *P. aeruginosa* ATCC 19142 biofilm on vertically suspended stainless steel and aluminum plates.

peroxide: 25,000 ppm sodium hypochlorite. Distilled water, 2,500 ppm hydrogen peroxide or 25,000 ppm sodium hypochlorite were used as controls. The test surfaces were submerged in 30 ml biocide complex or distilled water for 1 min, 5 min and 20 min contact time, followed by immersion in 30 ml D/E neutralizing broth for 20 min (Lindsay and Holey, 1999). Treated and control surfaces were each shaken in 20 ml phosphate buffer with 7.5 g sterile glass beads (3 mm) for 1 min to dislodge attached cells. Dislodged cells were enumerated, in duplicate, on Pseudomonas P agar using a spread plating technique. Plates were incubated for 24-48 h at 35˚C. All sanitizer efficacy experiments were conducted in triplicate for a total of at least two surveys.

2.9.6. Sanitizer Efficacy Calculation. The percent kill by each sanitizer was derived from the antilog of the log-percent kill using the following equation:

$$\log \% \text{ kill} = \log N - \log n$$

Where N is the count of untreated control cells and *n* is the cell count after sanitizer treatment (Lindsay and Von Holy, 1999). An effective sanitizer should reduce the initial planktonic cell

counts by 5 or more log units (i.e. 99.999%) and attached cell counts by 3 or more log (i.e. 99.9%) units in 30 s (Lindsay and Von Holy, 1999).

2.9.7. Microscopy Analysis. Surfaces exposed to *Pseudomonas aeruginosa* were sampled on day 6 as previously described, before and after sanitizer treatment, and prepared for Confocal Scanning Laser Microscopy (CSLM), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

2.9.7.1. SEM Sample Preparation. Biofilms on metal plates were fixed in 6% glutaraldehyde in 0.2 M cacodylate buffer for 1 h, rinsed three times in buffer for a total of 30 min, exposed to osmium tetroxide vapor for 1 h, briefly rinsed in 0.2 M cacodylate buffer, dehydrated in an ethanol series and air dried. The internal structure of the biofilm sample was exposed by fracturing the previously dried and observed sample. Aluminum and stainless steel SEM specimen stubs were overlain with adhesive and firmly pressed against the upper surface of the metal-coated biofilm. The stub was pulled straight up, breaking the biofilm between the upper and lower layers of the polysaccharide matrix to reveal the internal arrangement of bacterial cells. Sample plates were coated with gold/palladium in an Edwards S-150 sputter coater and the biofilm surface imaged with a Cambridge S-260 SEM. The average lengths of *Pseudomonas aeruginosa* cells were estimated by measuring the length of at least 10 individual cells before and after sanitizer treatment.

2.9.7.2. CSLM Sample Preparation. Metal plates were rinsed with distilled water and flooded with 150 ul of each component of the *Bac*light Bacterial Viability Kit™ (Molecular Probes, Leiden, The Netherlands), diluted to the manufacture's instructions (Lindsay et. al., 2002). Samples were kept in the dark at room temperature for 10 min, rinsed several times with sterile

distilled water and viewed at 63X/1.40-0.60 Oil CS HCX PL APO and 40X /1.25-0.75 Oil CS HCX PL APO on a Leica DM IRE2 inverted scope.

2.9.7.3. TEM Sample Preparation. Six days old *P. aeruginosa* biofilm growing on aluminum plates were treated with biocide for 20 min, the biocide neutralized with D/E neutralizing broth and the biofilm dislodged as previously described. Cells were centrifuged for 10 min at 8000 rpm and the pellet immediately fixed in 4% glutaraldehyde in 0.2 M cacodylate buffer for at least 30 min and rinsed twice in buffer. This step was followed by post fixation with 2% osmium tetroxide for 30 min. Cells were then filtered onto 0.2 µm polycarbonate filter and dehydrated with increasing concentrations of ethanol over 2 h. Cells were infiltrated with ethanol: LR White embedding resin (1:1) for 1 h, followed by 100 % LR White for 1 h. Cells were removed from the filter, embedded in LR White and polymerized at 60˚ overnight. Thin 60-90 nm sections were cut with a glass knife on a DuPont 5000 ultramicrotome, stained with Reynolds lead citrate and imaged with a JEOL 100CX TEM.

2.9.8. Statistical Analysis. The mean value of at least ten cell lengths was calculated after treatment with sanitizers. Means were then compared to each other and to the corresponding controls by multifactor analysis of variance (ANOVA; the SAS System for Windows 9.0, the SAS Institute, USA) at the 95% confidence level.

2.10. Fourier Transform Infrared Analysis of Biocide Treated *Pseudomonas aeruginosa* ATCC 19142 Biofilms.

2.10.1. Substrata Preparation. Stainless steel (Type 304) and aluminum (Alloy 3003) plates measuring 1 in wide by 2.5 in long by 3mm deep were polished to a mirror finish. At first, plates were coarse-polished using finer grades of emery paper (500, 1200 and 4000 mesh) mounted on a Struers LaboPol-5 lapping machine (Struers Inc, Denmark). Colloidal silica with an OP-NAP polishing cloth was later used for further polishing of the plates. The finishing step involved

using water and an oil based lubricant mixed with a suspension of polycrystalline diamonds (particle size, 3 μm).

2.10.2. Biofilm and Sample Preparation. An overnight culture of *Pseudomonas aeruginosa* ATCC 19142 was grown as previously described. A 2 ml inoculum of the overnight culture was inoculated into two separate 1 L beakers each containing 500 ml TSB and vertically suspended aluminum or stainless steel plates. Approximately 300 ml culture broth were removed and replaced with the same volume of fresh medium every two days for a total of eighteen days. Two samples each were retrieved on the 6^{th}, 12^{th} and 18^{th} day and rinsed once with 10 ml phosphate buffer. One of the two samples was submerged in 30 ml of the biocide complex for a 5 min contact time and later the biocide neutralized with 30 ml D/E neutralizing broth as described above. The second sample served as control and it was treated with phosphate buffer only. All samples were air dried and kept in a desiccator until all samples were collected. Samples were analyzed using FTIR spectrophotometry.

2.10.3. FTIR Analysis. Infrared spectroscopy has been employed for detecting and characterizing the activity of biofilm bacteria upon many types of substrate (Cheung et. al., 2000). A Spectrum 4000 FTIR spectrophotometer (Nicolet, Madison, WI) equipped with a narrow-band liquid nitrogen-cooled HgCdTe (MCT) detector was used for collecting the infrared spectrum. Spectra from 4000 to 700 cm^{-1} were collected in the reflectance mode with resolution of 4 cm^{-1}. Omnic software was used to express the ordinate as absorbance. Each spectrum was an average of 512 scans.

2.11. Decontamination of Bacterial Spores on Selected Office Materials

2.11.1. Anthrax Simulant. The organism used in this study was the spore-forming bacterium *Bacillus subtilis*. *Bacillus subtilis* is a Gram-positive, spore-forming, non- infectious organism that closely simulates *Bacillus anthracis*.

2.11.2. Biocide Preparation. The biocide was prepared on the same day of the experiment and consisted of a mixture of 2,500 ppm hydrogen peroxide: 25,000 ppm sodium hypochlorite with a final pH of 7. The final pH was adjusted with concentrated HCl. Also tested was 2,500 ppm hydrogen peroxide or 25,000 ppm sodium hypochlorite (pH 7) as controls. Before testing, all sanitizers and neutralizing solutions were equilibrated to room temperature (Lindsay and Von holey, 1999).

2.11.3. Materials Used in Test. Seven types of materials (Figures 7-13) characteristic of office environments with sizes ranging from 4 in x 4 in to 7.5 in x 7.5 in were autoclaved for 15 min at 121˚C prior to contamination with *Bacillus subtilis* spores.

2.11.4. Contamination Process. *Bacillus subtilis* spores were applied inside a microbiology-safe hood with internal air circulating system (Figure 14). A *Bacillus subtilis* aerosol spray with fine nozzle was directed perpendicularly to the surface of vertically suspended panel tests at a distance of about 14 in. Each test material received three sprays of ~10^8 spores ml^{-1} with a final deposition density of ~10^6 CFU in^{-2}.

2.11.5. Decontamination Sampling. The contaminated test materials were allowed to air-dry overnight at room temperature, approximately 17 hours. Each contaminated material was vertically placed against the wall under the safety hood and sprayed four times (a total volume of approximately 3 ml) with the biocide or controls at a distance of 14” as previously described. Untreated materials sprayed with distilled water served as controls. Samples of decontaminated

Figure 7. Wood floor tile.

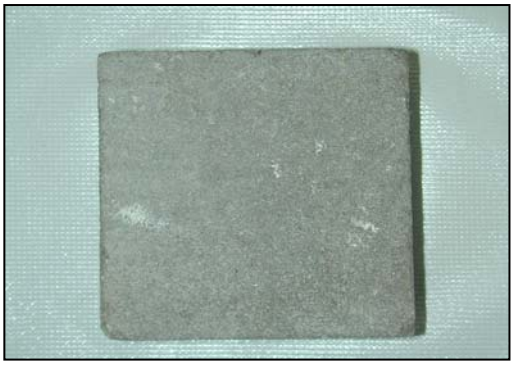


Figure 8. Old concrete block.

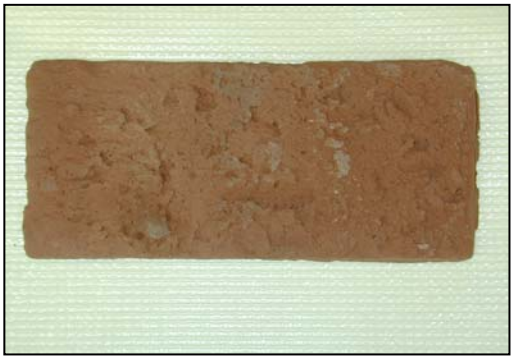


Figure 9. Rough surface tile.

Figure 10. Acoustic ceiling tile.

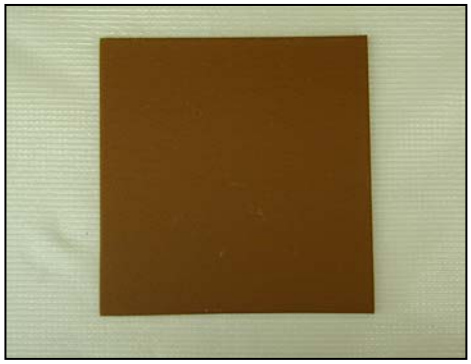


Figure 11. Smooth surface tile.

Figure 12. Cork tile.

Figure 13. Woven carpet.

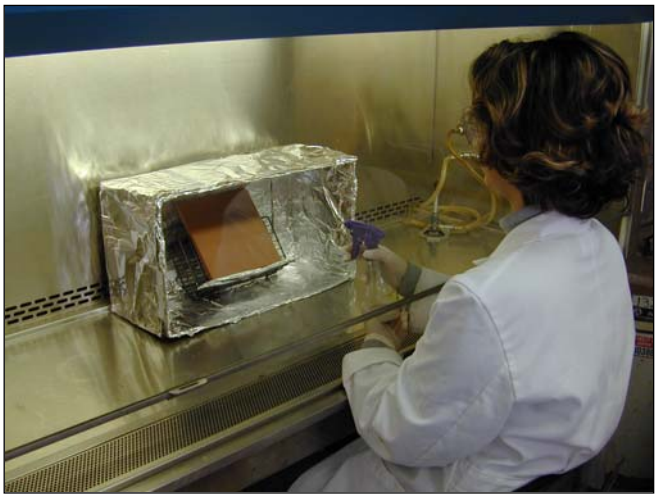


Figure 14. Contamination of test materials with *Bacillus subtilis* spores.

materials were taken at two different locations, at time zero and after 5 or 10 min (when necessary) contact time. A sterile cotton tip applicator was rolled back and forth within a 1.25 in x 1.25 in area and placed into a test tube containing 5 ml of D/E neutralizing broth. The test tubes containing the swab samples were analyzed immediately for spore survival.

2.11.6. Microbiological Assay. The concentration of *Bacillus subtilis* spores was determined on Plate Count Agar (PCA). The spore suspension was serially diluted on Butterfield's phosphate buffer, pH 7. Aliquots of the appropriate dilutions of each sample were plated in triplicates. All plates were incubated at 37°C for 24-48 hours.

2.11.7. Statistical Analysis. The mean value of duplicate plate counts of *B. subtilis* spores survival following sanitizer treatment was calculated after two surveys. Means were then compared to each other and to the corresponding controls by multifactor analysis of variance (ANOVA; the SAS System for Windows 9.0, the SAS Institute, USA) at the 95% confidence level.

2.12. Effectiveness of Biocidal Complex at a Poultry Processing Facility

2.12.1. Biocide Complex Preparation. Solutions of hydrogen peroxide: sodium hypochlorite (15 ppm: 150 ppm, 25 ppm: 250 ppm and 35 ppm: 350 ppm) were prepared on site from 30% and 6% stock solutions, respectively. A biocide concentrate was first prepared and further diluted to the desired concentration by adding 20 Gal of water and 5 Gal of ice by weight.

2.12.2. Evaluation of Sanitizer Efficacy. This study was performed at Sanderson Farms' processing facility in Laurel, MS. A total of 10 eviscerated and non-chilled whole bird carcasses, weighing an average of 7 lbs were placed in 5 different 50 Gal tanks to simulate the chilling process. Three tanks contained each a different hydrogen peroxide: sodium hypochlorite complex combination (12.5 ppm: 125 ppm, 25 ppm: 250 ppm or 35 ppm: 350 ppm, respectively). The other two tanks which served as controls contained chilled water or trisodium phosphate (TSP). Trisodium phosphate is the current and only antimicrobial agent use in the chiller at this poultry processing facility. Each tank was stirred for 5 min before and every 20 min after addition of bird carcasses to achieve an even distribution of biocide components. Bird

carcasses were allowed to reached an internal temperature of 40˚F and sampled after 1 ½ h to simulate the typical exposure time in a poultry processing facility.

2.12.3. Microbial Analysis. After 1 ½ h exposure to sanitizers, bird carcasses were aseptically removed and each placed in a sterile stomacher bag to which 500 ml phosphate buffer, pH 7, were added. The bags and their contents were shaken for 1 min and approximately 400 ml of the liquid were collected for analysis and immediately stored in an ice box. Samples were serially diluted in phosphate buffer, spread-plated onto Eosin Methylene Blue (EMB) agar plates (Sparks, MD) for the detection of *Escherichia coli* and *Salmonella spp*. and pour plated onto PCA (5g tryptone; 2.5 g yeast extract; 1 g dextrose and 15 g agar / L) agar plates for total aerobic counts. EMB plates were incubated at 35˚C and PCA plates at 30˚C for 24-48 h. All samples were analyzed the same day they were collected.

2.12.4. Statistical Analysis. The mean value of duplicate plate counts for *Escherichia coli*, *Salmonella spp*. and total bioburden was calculated for each treatment. Means were then compared to each other and to the corresponding controls by multifactor analysis of variance (ANOVA; the SAS System for Windows 9.0, the SAS Institute, USA) at the 95% confidence level.

2.13. Effectiveness of Biocidal Complex at a Sugar Mill

2.13.1. Preparation of Biocidal Complex. Three strengths of the hydrogen peroxide and sodium hypochlorite biocide complex (5X, 20X, 50X) at a concentration of 30 ppm hydrogen peroxide: 200 ppm sodium hypochlorite were prepared and tested on site at Cora Texas sugar mill, LA. The order of addition of each biocide component was kept constant, with hydrogen peroxide being added to sodium hypochlorite followed by distilled water.

2.13.2. Microbial Analysis. An 8 in x 40 in section of cane washing equipment covered with a thick biofilm (~5mm) located behind the wash tables at Cora Texas sugar mill was used as the testing area. This area was divided into four sections (control, 5X, 20X and 50X) of approximately 8" x 6" each (Figure 15). A stainless steel shield (8 in x 31 in x 6 in) was built to protect the area from dripping water. The biocide complex was sprayed twice (~ 5.4 ml) (Hudson sprayer Model 60136, Hasting, MN) 12 in away from the surface area. Distilled water, hydrogen peroxide (50X) or sodium hypochlorite (50X) was sprayed as control. A 1.56 in^2 area was swabbed with a cotton tip applicator before and after treatment with biocide complex for contact times of 0.30 min, 15 min and 30 min. Samples were stored on ice in 5 ml phosphate buffer solutions and analyzed for total aerobic count on the same day of the experiment. Samples were plated on PCA in duplicates and incubated for 24-48 h at 30˚C.

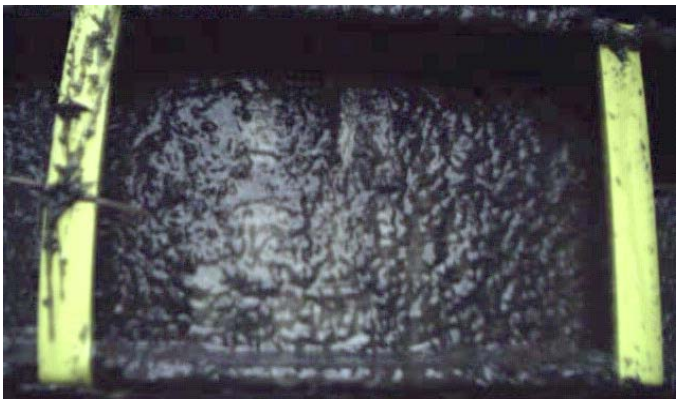


Figure 15. A section of a wash table covered with biofilm at Cora Texas mill, LA.

2.14. Evaluation of IB Biocide as a Hard Surface Disinfectant.

Evaluation of IB biocide was performed by following the Standard Operating Procedures (SOPs) for "Neutralization Confirmation Procedure for Products Evaluated with the AOAC

Sporicidal Activity Test" (SOP number: MB-12-00) and for "AOAC Sporicidal Activity Test (*Bacillus* sp.)" (SOP number: MB-15-00) as described by EPA (EPA, 2004). These SOPs are suitable for determining the presence or absence of sporicidal activity of liquid sporicidal agents against spores from the genus *Bacillus*.

2.14.1. SOP for Neutralization Confirmation Procedure for D/E Neutralizing Broth Evaluated with the AOAC Sporicidal Activity Test against *Bacillus subtilis* ATCC 19659 Spores

2.14.1.1. Sporicidal Agent Sample Preparation. A stock solution of IB biocide at a concentration of 4% sodium hypochlorite and 0.4% hydrogen peroxide was prepared and stored in a sterile glass bottle. Approximately 10 ml aliquots of the ready to use IB biocide was dispensed into 20 mm x 150 mm disposable test tubes and allowed to equilibrate to room temperature for 10 min.

2.14.1.2. Preparation of Inoculum. Serial ten-fold dilutions of a *B. subtilis* ATCC 19659 spore suspension were prepared. Approximately, a 1 ml of the spore suspension was transferred into 9 ml of sterile distilled water. Five dilutions (1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8} and 1×10^{-9}) were prepared and used to inoculate D/E neutralizing broth and subculture media tubes described below. The dilution series were based on an estimate of 10^{8} spores/ml for the undiluted suspension. The pour plate method was used to estimate CFUs/ml of each of the five dilutions in duplicate on TSA.

2.14.1.3. Product Sample Preparation. A set of five uninoculated porcelain penicylinders (8±1 mm, 6±1 mm ID, 10±1 mm length) (Presque Isle Cultures, Erie, PA) was placed into a test tube containing 10 ml of IB biocide for a contact time of 10 min. Each carrier was then aseptically transferred into 5 tubes containing 10 ml of D/E neutralizing broth for 30 min. All transfers were made within 2 min. This set of neutralizer tubes (a total of five) represented the neutralizer-

primary subculture treatment. After 30 min, each carrier was aseptically removed from the neutralizing solution and transferred into a tube containing 10 ml of thioglycollate medium. This set of tubes (a total of five) represented the secondary subculture treatment. Each set of the neutralizer-primary subculture treatment and the secondary subculture treatment was inoculated with 1 ml of each of the five inoculum dilutions (1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8} and 1×10^{-9}) as indicated in Table 5. The target number of cells to be delivered was 5-100 CFUs/ml. Controls of inoculated and uninoculated D/E neutralizing and thioglycollate broth were also prepared. All test tubes were incubated for 21 days at 37˚C. Tubes showing no growth after 21 days were heat shocked for 20 min at 80˚C and re-incubated for 72 h. Results were reported as + for growth and 0 for no growth.

2.14.2. SOP for AOAC Sporicidal Activity Test of IB Biocide against *Bacillus subtilis* ATCC 19659 Spores

2.14.2.1. Storage of Pre-inoculated Porcelain Carriers. Porcelain penicylinders pre-inoculated with *B. subtilis* ATCC 19659 spores were purchased from Presque Isle Cultures (Erie, PA) and stored under vacuum upon arrival.

2.14.2.2. Spore Load Determination of Porcelain Penicylinders Inoculated with *B. subtilis* Spores. Four randomly selected carriers were each placed into a 20 x 150 mm tube containing 10 ml sterile distilled water and vortex-mixed for 7 min. Dilutions of each spore suspension were prepared by transferring 1 ml aliquots to tubes containing 9 ml sterile distilled water. Each spore suspension was diluted out to 10^{-7} and 1 ml of dilutions 10^{-4} to 10^{-7} plated by the pour plate method on TSA. Each dilution was plated in duplicate and incubated for 24 – 48 h at 37˚C.

2.14.2.3. Testing Procedure. Sixty inoculated porcelain penicylinders and three uninoculated porcelain penicylinders (control) were sequentially transferred at 2 min intervals in groups of five and one group of three (control) to thirteen tubes of IB biocide using a sterile loop for a 10.

Table 5. Seeding treatments and control groups for the D/E Neutralization Confirmation Test.

Neutralizer-primary*^ Subculture Treatment	**Secondary# Subculture Treatment**	**Neutralizer-primary Subculture Inoculated Control**	**Secondary Subculture Inoculated Control**	**Neutralizer-Primary & Secondary Subculture Uninoculated Controls**
1 ml 10^{-5}----tube 1	1 ml 10^{-5}----tube 1	1 ml 10^{-5}----tube 1	1 ml 10^{-5}----tube 1	
1 ml 10^{-6}----tube 2	1 ml 10^{-6}----tube 2	1 ml 10^{-6}----tube 2	1 ml 10^{-6}----tube 2	D/E neutralizing broth
1 ml 10^{-7}----tube 3	1 ml 10^{-7}----tube 3	1 ml 10^{-7}----tube 3	1 ml 10^{-7}----tube 3	Thioglycollate broth
1 ml 10^{-8}----tube 4	1 ml 10^{-8}----tube 4	1 ml 10^{-8}----tube 4	1 ml 10^{-8}----tube 4	
1 ml 10^{-9}----tube 5	1 ml 10^{-9}----tube 5	1 ml 10^{-9}----tube 5	1 ml 10^{-9}----tube 5	

* = 1 x 10^{-5} through 1 x 10^{-9} based on an approximation starting from a suspension of 10^{8} spores/ml.

^ = Neutralizer-primary subculture treatment: D/E neutralizing broth.

= Secondary subculture treatment: Thioglycollate broth.

min contact time. Carriers were transferred one at a time into separate tubes containing 10 ml D/E neutralizing broth and gently mixed to facilitate adequate mixing and efficient neutralization. After all transfers were completed; each carrier was transferred again to tubes containing 10 ml of thioglycollate media (secondary subculture). Neutralizer tubes (63 tubes) and thioglycollate tubes (63 tubes) were incubated for 21 days at 37˚C. Three unopened, uninoculated neutralizer media and three unopened, uninoculated thioglycollate media tubes were incubated with the sample tubes for 21 days at 37˚C also as controls. A positive result in either the neutralizer or secondary subculture tube was considered a positive result for a carrier set. If no growth was observed after 21 days, the tubes were heat-shocked at 80 ± 1˚C for 20 min and incubated an additional 72 h at 37˚C. Results were reported as + for growth and 0 for no growth. The above test was repeated twice for contact times of 30 min and 60 min.

2.14.2.4. Confirmatory Testing. A minimum of three positive carrier sets per test, if available, were confirmed using gram staining and general growth media (TSA). If there were less than three positive carrier sets, then each carrier set was confirmed. For a test with greater than 20 positive carrier sets, at least 20% of positive samples were confirmed by gram staining and general growth media. If both tubes were positive in a carrier set, only one tube was selected for confirmatory testing.

3. RESULTS

3.1. Antimicrobial Activity of a Hydrogen Peroxide and Sodium Hypochlorite Complex

3.1.1. Antimicrobial Activity against Bacterial Cells and Bacterial Spores

3.1.1.1. Minimum Inhibitory Concentrations and Fractional Inhibitory Concentrations of Bacterial Cells. Any ratio for the combined reaction of hydrogen peroxide and sodium hypochlorite mentioned anywhere on this thesis is expressed as the ratio of hydrogen peroxide: sodium hypochlorite. Minimum Inhibitory Concentrations (MICs) for hydrogen peroxide (H_2O_2), sodium hypochlorite (NaOCl) and combinations against a range of bacterial cells are presented in Table 6. Overall, sodium hypochlorite required 4 times the concentration of hydrogen peroxide for effectiveness against bacterial cells. Hydrogen peroxide MIC's ranged from 50 ppm to 250 ppm. Of the organisms tested, *X. maltophilia*, *L. monocytogenes*, *V. parahaemolyticus* and *V. cholera* were most susceptible to hydrogen peroxide and *X. maltophilia* and *V. parahaemolyticus* were most susceptible to sodium hypochlorite. *B. subtilis*, *B. pumilus* RJ 0055 and *B. cereus* were resistant to either hydrogen peroxide or sodium hypochlorite with MICs of 250 ppm and 1000 ppm, respectively. The MICs of the combination required from one sixth to one half the concentration of hydrogen peroxide and from one twentieth to one half that of sodium hypochlorite normally required to kill the organisms tested. Fractional Inhibitory Concentrations (FICs) of biocidal complex for bacterial cells are shown in Table 7. FIC values ranged from 0.47 to 0.85, which are indicative of a synergism between hydrogen peroxide and sodium hypochlorite FIC values of less than one indicate that a synergistic effect exists between components (Berenbaum, 1978).

Table 6. Minimum Inhibitory Concentrations (MIC) for hydrogen peroxide, sodium hypochlorite and the combination against bacterial cells in broth culture.

Organism	MIC (ppm)			
	Hydrogen Peroxide	Sodium Hypochlorite	In Complex	
			Hydrogen Peroxide	Sodium Hypochlorite
S. typhimurium ATCC 14028	100	200	50	50
E. coli B ATCC 23226	100	200	50	50
***P. aeruginosa* ATCC 19142**	150	500	25	300
X. maltophilia	50	100	35	15
L. mesenteroides B 512F	60	350	20	180
B. subtilis	250	1,000	50	600
B. pumilus RJ 0055	250	1,000	150	100
B. cereus	250	1,000	150	100
B. anthracis	100	500	25	250
L. monocytogenes	50	500	10	250
***C. jejuni subsp. Jejuni* ATCC 33560**	150	500	120	25
V. cholera	50	150	35	20
V. parahaemolyticus	50	100	25	20

Table 7. Fractional Inhibitory Concentrations (FIC) for biocidal complex against bacterial cells in broth culture.

Organism	Biocidal Complex FIC[a]
S. typhimurium ATCC 14028	0.75
E. coli B ATCC 23226	0.75
***P. aeruginosa* ATCC 19142**	0.77
X. maltophilia	0.85
L. mesenteroides B 512F	0.85
B. subtilis	0.80
B. pumilus RJ 0055	0.70
B. cereus	0.70
B. anthracis	0.75
L. monocytogenes	0.70
***C. jejuni subsp. Jejuni* ATCC 33560**	0.47
V. cholera	0.83
V. parahaemolyticus	0.70

[a]The FIC was calculated from the sum of the ratios of the MIC of each biocide used in combination divided by its MIC when used alone.

3.1.1.2. Minimum Inhibitory Concentrations and Fractional Inhibitory Concentrations of Bacterial Spores. Minimum inhibitory concentrations for bacterial spores, in distilled water or broth culture, treated for 5 min or 24 h with sanitizers are presented in Table 8. In broth culture, sodium hypochlorite was effective against *B. subtilis* or *B. pumilis* RJ OO55 spores at concentrations of 40,000 ppm or 35,000 ppm and hydrogen peroxide at 50,000 ppm or 40,000 ppm. *Bacillus anthracis* spores were sensitive to both hydrogen peroxide and sodium hypochlorite at 10,000 ppm. MIC's ranged from one twentieth to one half and from one fourth to one half that of hydrogen peroxide and sodium hypochlorite, respectively. The MIC ratio of the combination was 0.1 (2,500 ppm hydrogen peroxide) for *B. subtilis*, *B.cereus* and *B. pumilis* RJ OO55 spores and 2 (5,000 ppm hydrogen peroxide) for *B. anthracis* spores. MIC values of >500,000 ppm and 60,000 ppm were obtained for hydrogen peroxide and sodium hypochlorite against *B. subtilis* spores in distilled water, respectively. The MIC ratio for the combination was 0.1 (4,000 ppm hydrogen peroxide). FIC values, in both broth culture and distilled water, ranged from 0.68 to 0.85.

3.1.2. Ratio Effect on Biocidal Complex Activity. Biocidal complex (hydrogen peroxide: sodium hypochlorite) solutions were prepared at different ratios (1:1, 1:5, 5:1, 10:1, 1:10, 1:20, 1:50 and 1:100) and their antimicrobial activity tested against *B. subtilis* spores in distilled water. Spore survival as a function of ratio is presented in Figure 16. Biocidal complex solutions at ratios of 1:1, 5:1 and 10:1 reduced spore counts by less than 0.5 log units. Solutions with a ratio of 1:5 reduced counts by 3.8 log units. Increasing the ratio to 1:20, 1:50 and 1:100 resulted in log reductions of 5.5, 4.2 and 4.2 log, respectively. Only a 1:10 ratio yielded undetectable spore counts. The FIC values of the solutions tested were less than 1 and ranged from 0.15 (10:1 ratio) to 0.85 (1:5 ratio).

Table 8. Minimum Inhibitory Concentrations (MIC) and Fractional Inhibitory Concentrations (FIC) for hydrogen peroxide, sodium hypochlorite and the combined reaction against bacterial spores in broth culture or distilled water.

Organism	MIC (ppm)				FIC[a]
			In Complex		
	Hydrogen Peroxide	Sodium Hypochlorite	Hydrogen Peroxide	Sodium Hypochlorite	
In broth culture:					
B. anthracis	10,000	10,000	5,000	2,500	0.75
B. cereus	50,000	40,000	2,500	25,000	0.68
B. subtilis	50,000	40,000	2,500	25,000	0.68
B. pumilus RJ 0055	40,000	35,000	2,500	25,000	0.85
In distilled water:					
B. subtilis*	> 500,000	60,000	4,000	40,000	0.68

[a]The FIC was calculated from the sum of the ratios of the MIC of each biocide used in combination divided by its MIC when used alone.

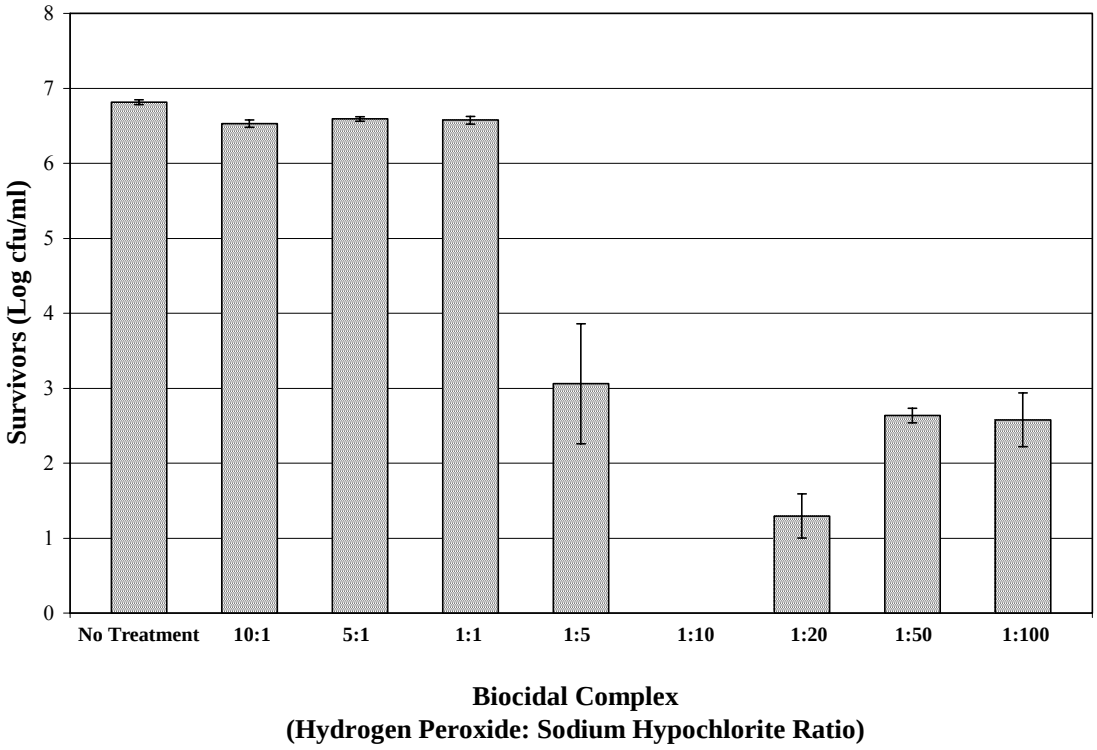


Figure 16. Ratio effect of biocidal complex on spore survival. *B. subtilis* spores were treated for 5 min with biocidal complex solutions prepared at different ratios of hydrogen peroxide: sodium hypochlorite. Ratios tested included (a) 1:1 (40,000 ppm H_2O_2), (b) 1:5 (10,000 ppm H_2O_2), (c) 5:1 (50,000 ppm H_2O_2), (d) 10:1 (40,000 ppm H_2O_2), (e) 1:10 (4,000 ppm H_2O_2), (f) 1:20 (2,000 ppm H_2O_2), (g) 1:50 (800 ppm H_2O_2) and (h) 1:100 (400 ppm H_2O_2). Error bars show the standard error of the mean.

3.1.3. Synergy of Sodium Peroxide with Sodium Hypochlorite. MICs and FICs of the biocidal complex, combinations of sodium peroxide and sodium hypochlorite as well as each biocide component against vegetative *P. aeruginosa* ATCC 19142 cells are presented in Table 9. The MIC for hydrogen peroxide or sodium peroxide was 150 ppm, where as sodium hypochlorite produced an MIC of 500 ppm. MICs ratio for the biocidal complex was 0.08 (25 ppm hydrogen peroxide). The sodium peroxide and sodium hypochlorite combination generated an MIC ratio of 0.2 (50 ppm sodium peroxide). The biocidal complex generated a lower FIC value (0.77) than the sodium peroxide and sodium hypochlorite combination (0.83). Nevertheless, both combinations were synergistic.

3.2. Antimicrobial Activity of Biocidal Complex against Spores on Stainless Steel

3.2.1. Time Exposure and Temperature Effects. Effectiveness of the biocidal complex against *B. subtilis* spores dried on stainless steel plates was compared to that of sodium hypochlorite at 4˚C, 24˚C and 37˚C for 1 min, 5 min, 15 min and 30 min contact times. The standard biocide was tested at a ratio of 1:10 (2,500 ppm hydrogen peroxide) at an alkaline pH. The pH of both biocidal complex and sodium hypochlorite solutions was 10.8 and 10.9, respectively. Untreated stainless steel plates averaged 6.0 log CFU cm^{-2}. Treatments with biocidal complex solutions stored at 4˚C (Figure 17), 24˚C (Figure 18) or 37˚C (Figure 19) killed 11%, 12% or 27% of the *B. subtilis* spores within 1 min, respectively. Reductions of less than 4% were observed for sodium hypochlorite at each temperature within 1 min. A 5 min exposure to biocidal complex reduced spores by 49%, 60% or 73% at 4˚C, 24˚C or 37˚C, respectively. These were 3.0, 3.8 and 4.6 log CFU cm^{-2} reductions from initial load. Only 47%, 51% or 49% of spores were killed after 5 min treatment with sodium hypochlorite at 4˚C, 24˚C or 37˚C, which resulted in 3.0, 3.2 and 3.1 log CFU cm^{-2} reductions, respectively.

Table 9. Minimum Inhibitory Concentrations (MIC) and Fractional Inhibitory Concentrations (FIC) for hydrogen peroxide, sodium peroxide, sodium hypochlorite and combined reactions against *Pseudomonas aeruginosa* ATCC 19142 cells in broth culture.

Chemical Compounds	**MIC (ppm)**	**FIC[a]**
Hydrogen Peroxide	150	-
Sodium Peroxide	150	-
Sodium Hypochlorite	500	-
Hydrogen Peroxide: Sodium Hypochlorite	25: 300	0.77
Sodium Peroxide: Sodium Hypochlorite	50: 250	0.83

[a]The FIC was calculated from the sum of the ratios of the MIC of each biocide used in combination divided by its MIC when used alone.

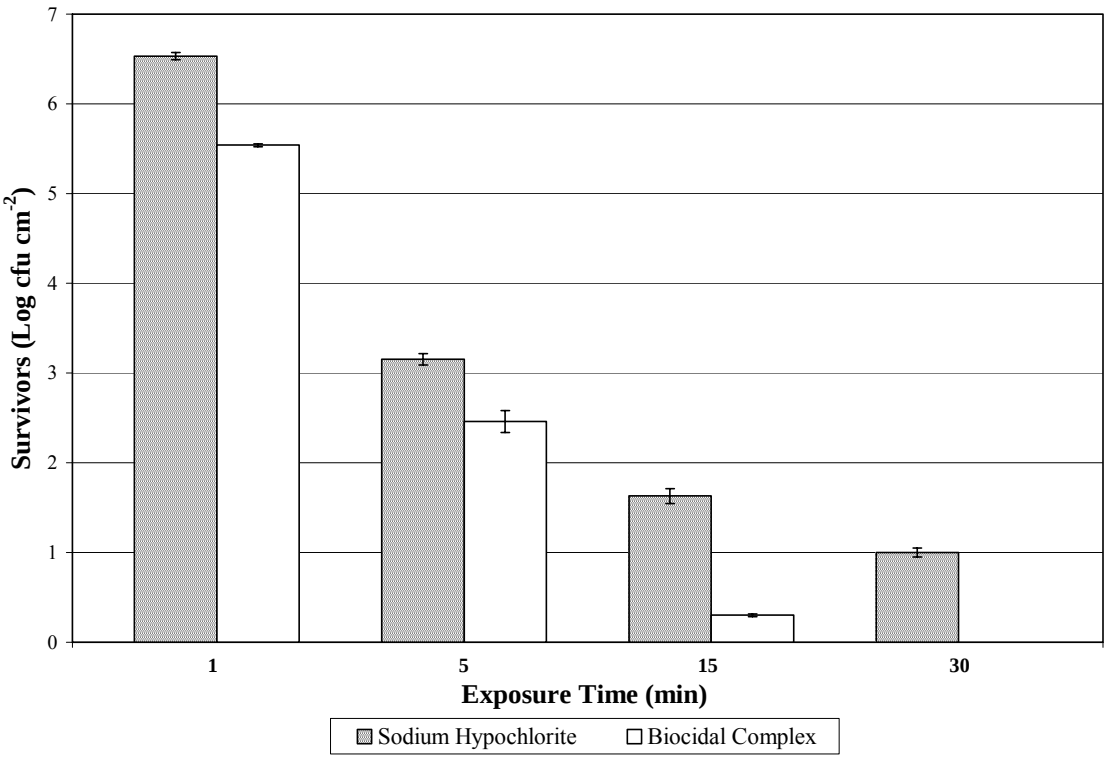


Figure 17. Effect of exposure time of biocide against *B. subtilis* spore-contaminated stainless steel plates at 4˚C. Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at 4˚C at a ratio of 1:10 (2,500 ppm H_2O_2) and contact times of 1 min, 5 min, 15 min, and 30 min . A solution of sodium hypochlorite, as control, was tested at a concentration of 25,000 ppm. Initial spore population on stainless steel plates prior to treatment with biocide averaged 6.0 log CFU cm^{-2}. Error bars show the standard error of the mean.

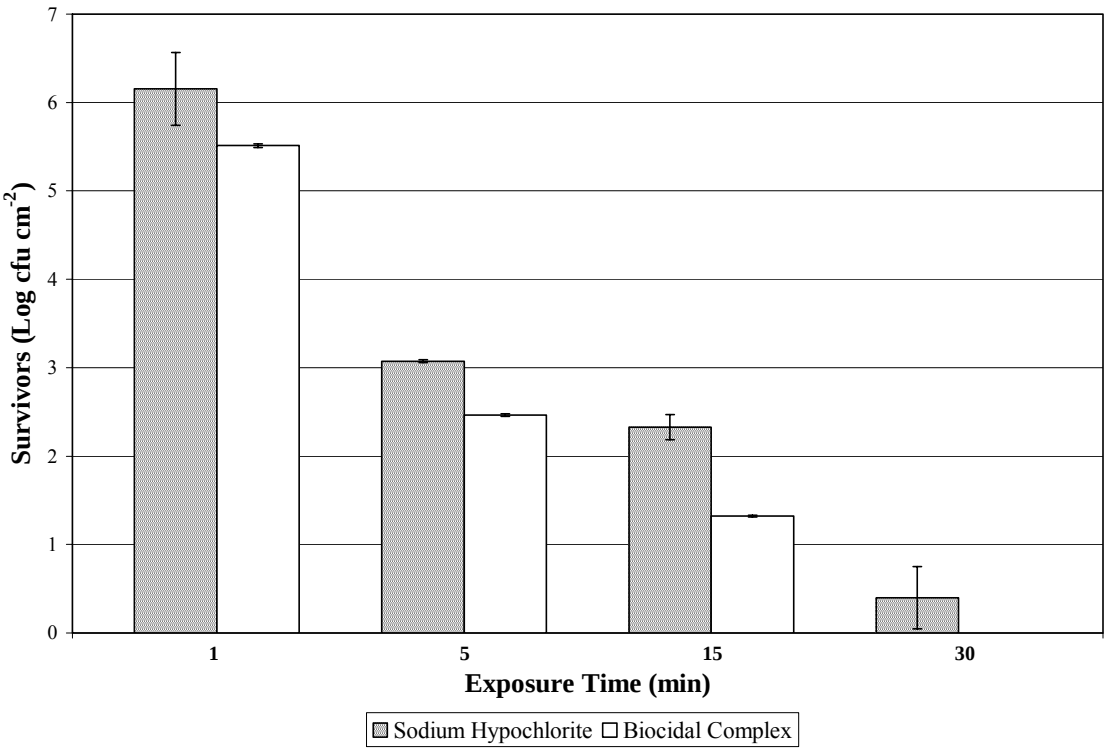


Figure 18. Effect of exposure time of biocide against *B. subtilis* spore-contaminated stainless steel plates at 24˚C Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at 24˚C at a ratio of 1:10 (2,500 ppm H_2O_2) and contact times of 1 min, 5 min, 15 min, and 30 min . A solution of sodium hypochlorite, as control, was tested at a concentration of 25,000 ppm. Initial spore population on stainless steel plates prior to treatment with biocide averaged 6.0 log CFU cm^{-2}. Error bars show the standard error of the mean.

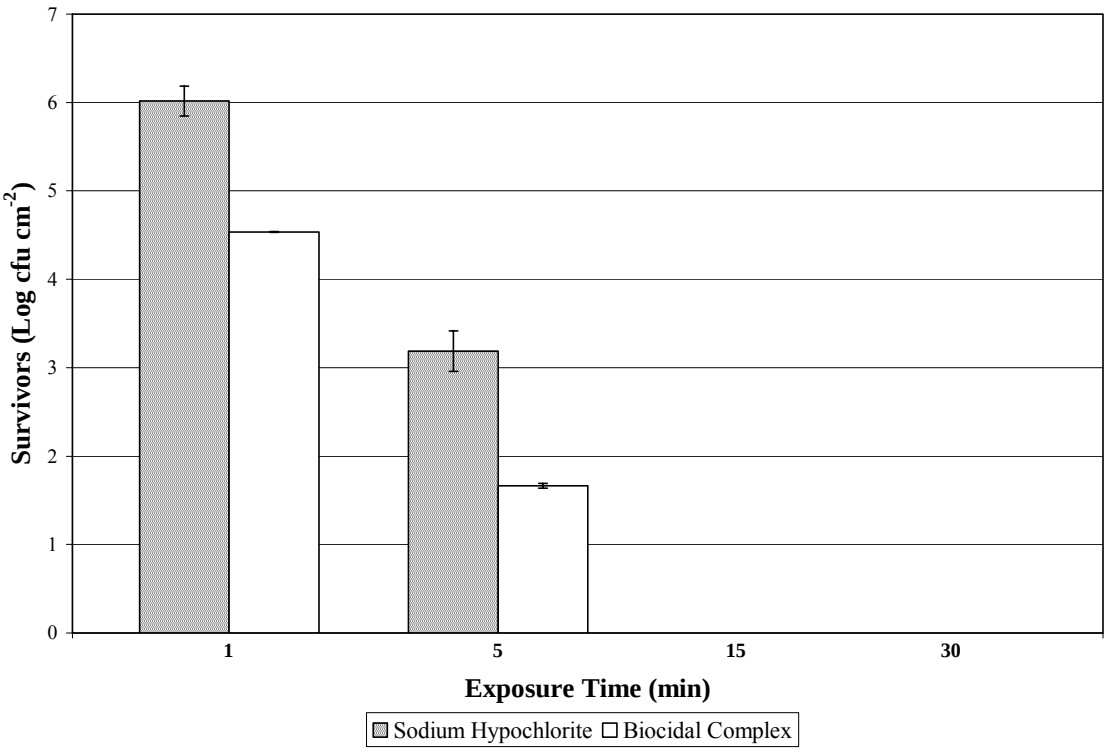


Figure19. Effect of exposure time of biocide against *B. subtilis* spore-contaminated stainless steel plates at 37˚C. Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at 37˚C at a ratio of 1:10 (2,500 ppm H_2O_2) and contact times of 1 min, 5 min, 15 min, and 30 min. A solution of sodium hypochlorite, as control, was tested at a concentration of 25,000 ppm. Initial spore population on stainless steel plates prior to treatment with biocide averaged 6.0 log CFU cm^{-2}. Error bars show the standard error of the mean.

B. subtilis spores were completely eliminated by biocidal complex after 15 min at 37˚C or 30 min regardless of temperature. No spores were recovered after 15 min or 30 min treatment with sodium hypochlorite at 37˚C. Temperature had little influence on the effectiveness of sodium hypochlorite against *B. subtilis* spores, however, the biocidal complex showed about a 2x greater kill at the higher temperature (Figure 20).

3.2.2. pH Effect. *B. subtilis* spore-contaminated stainless steel plates were treated with biocidal complex or sodium hypochlorite solutions at pH 11 or pH 6.5, at 4˚C for 15 min. The results are summarized in Figure 21. Treatment with biocidal complex at pH 11 killed 97% of the spores, where as treatment with sodium hypochlorite resulted in a 74% kill. The log kill difference from untreated samples was 6 and 4.5 log CFU cm^{-2} for biocidal complex and sodium hypochlorite, respectively. A 100% kill was observed for both biocidal complex and sodium hypochlorite at the lower pH.

3.3. Biocide Stability

3.3.1. Spectrophotometer Scans. The stability of the biocidal complex at a 1:10 ratio (2,500 ppm hydrogen peroxide), along with that of hydrogen peroxide or sodium hypochlorite, at different pH's (pH 11 and pH 6.5), temperature's (4˚C, 24˚C and 37˚C), and in the presence or absence of U/V light was monitored by conducting differential wave scans (200 nm to 450 nm) on each sample, calculating the change in area of each scan over a 42 day period and correlating this with biological effectiveness against bacterial spores. Signature scans for the biocidal complex, hydrogen peroxide and sodium hypochlorite are presented in Figure 22. Biocidal complex and sodium hypochlorite solutions scanned against a water baseline (Figure 22A) showed their highest peak around 300 nm. The area below the curve of the biocidal complex scan (200 nm to 400 nm) was smaller than the area below the curve of the sodium hypochlorite

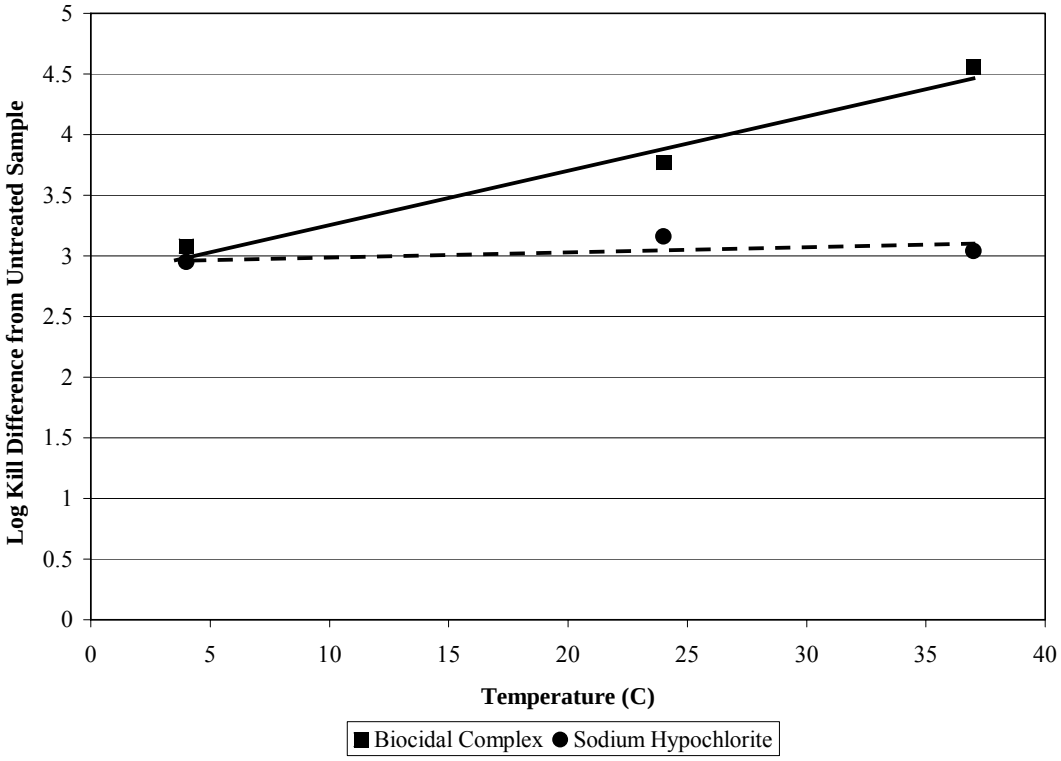


Figure 20. Temperature effect of biocide against *B. subtilis* spore-contaminated stainless steel plates with a 5 min exposure. Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at a ratio of 1:10 (2,500 ppm H_2O_2) at 4˚C, 24˚C and 37˚C for a 5 min contact time. A solution of sodium hypochlorite, as control, was tested at a concentration of 25,000 ppm. Initial spore population on stainless steel plates prior to treatment with biocide averaged 6.0 log CFU cm^{-2}. Error bars show the standard error of the mean.

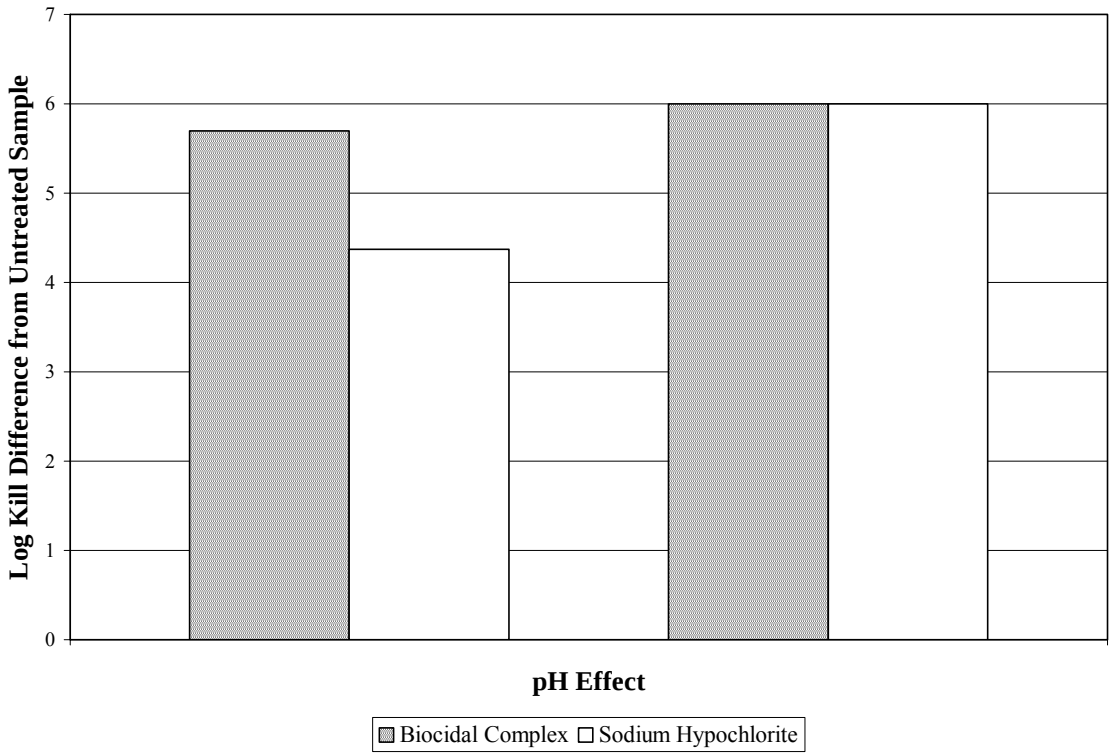
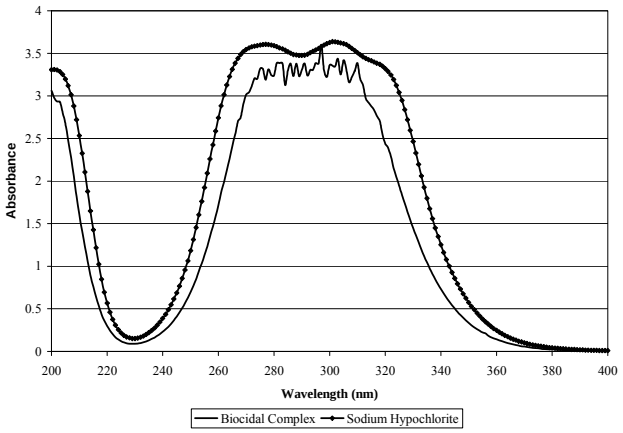
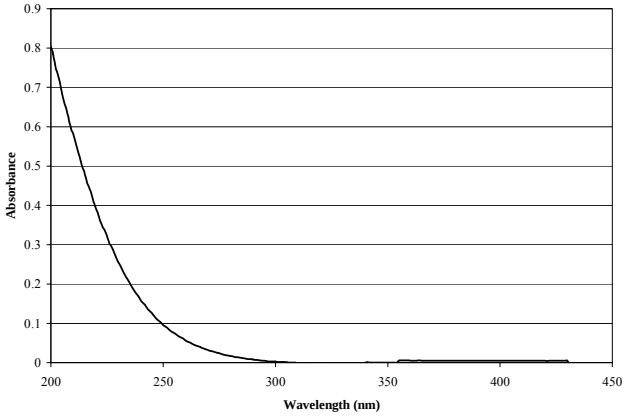


Figure 21. pH effect of biocide against *B. subtilis* spore-contaminated stainless steel plates with a 15 min exposure at 4˚C Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at a ratio of 1:10 (2,500 ppm H_2O_2) at 4˚C for a 15 min contact time. A solution of sodium hypochlorite, as control, was tested at a concentration of 25,000 ppm. The pH of each biocide was adjusted to 6.5 with concentrated HCl. Initial spore population on stainless steel plates prior to treatment with biocide averaged 6.0 log CFU cm^{-2}.

A.



B.



C.

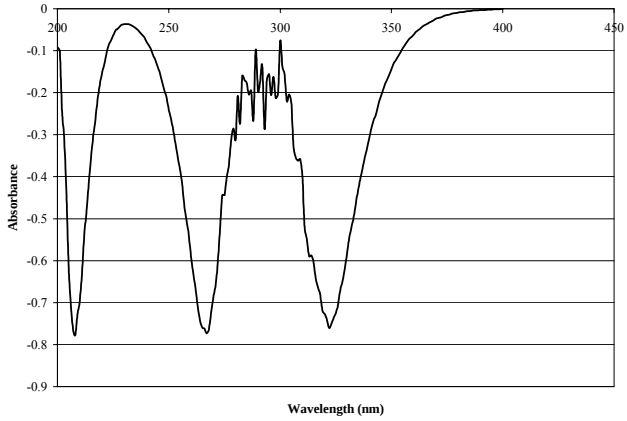


Figure 22. Signature scans for (a) biocidal complex and sodium hypochlorite against a water baseline, (b) hydrogen peroxide against a water baseline and (c) biocidal complex against a sodium hypochlorite baseline. Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at a ratio of 1:10 (2,500 ppm H_2O_2), sodium hypochlorite at 25,000 ppm and hydrogen peroxide at 2,500 ppm.

scan (200 nm to 400 nm) with ∫Absorbance (λ)(d)λ = 276 and ∫Absorbance(λ)(d)λ = 350, respectively. The calculated area for the hydrogen peroxide differential wave scan (200 nm to 400 nm) was ∫Absorbance (λ)(d)λ = 20 (Figure 22B). The difference between the biocidal complex integral and the sodium hypochlorite integral was ∫Absorbance (λ)(d)λ = 74. This difference closely resembles the area below the curve of the biocidal complex scan (200 nm to 400 nm) scanned against a sodium hypochlorite baseline, which was ∫Absorbance(λ)(d)λ = 55 (Figure 22C).This signature scan possibly represents "the semi- stable complex" that results from the reaction of hydrogen peroxide and sodium hypochlorite at a 1:10 ratio. Figure 23 shows the degradation of this semi-stable complex from a biocidal complex solution (pH 10.8) stored in the presence of U/V light for a period of 26 days at 24˚C. The degradation or loss of activity of biocidal complex, sodium hypochlorite and hydrogen peroxide was monitored by conducting differential wave scans against a water baseline.

3.3.2. Effect of Temperature, pH and U/V Light. The half-lives of the biocidal complex, hydrogen peroxide and sodium hypochlorite are summarized in Table 10. Hydrogen peroxide, sodium hypochlorite and biocidal complex solutions degraded more rapidly at higher temperatures, with sodium hypochlorite showing the greatest loss (Figure 24). Half-lives at 4˚C for sodium hypochlorite, hydrogen peroxide and biocidal complex were 708 days, 220 days and 123 days, respectively. A drop of 24% and 36% loss of activity was observed for biocidal complex solutions stored at temperatures of 24˚C and 37˚C, respectively. Half-lives of sodium hypochlorite solutions decreased by 75% or 76% when stored at 24˚C or 37˚C. Storing hydrogen peroxide at 24˚C or 37˚C reduced its half-life by 40% or 45%. The biocidal complex stability paralleled that of hydrogen peroxide, not sodium hypochlorite. The half-lives of the antimicrobial agents were reduced by acidic conditions (Figure 25). The half-life of hypochlorite,

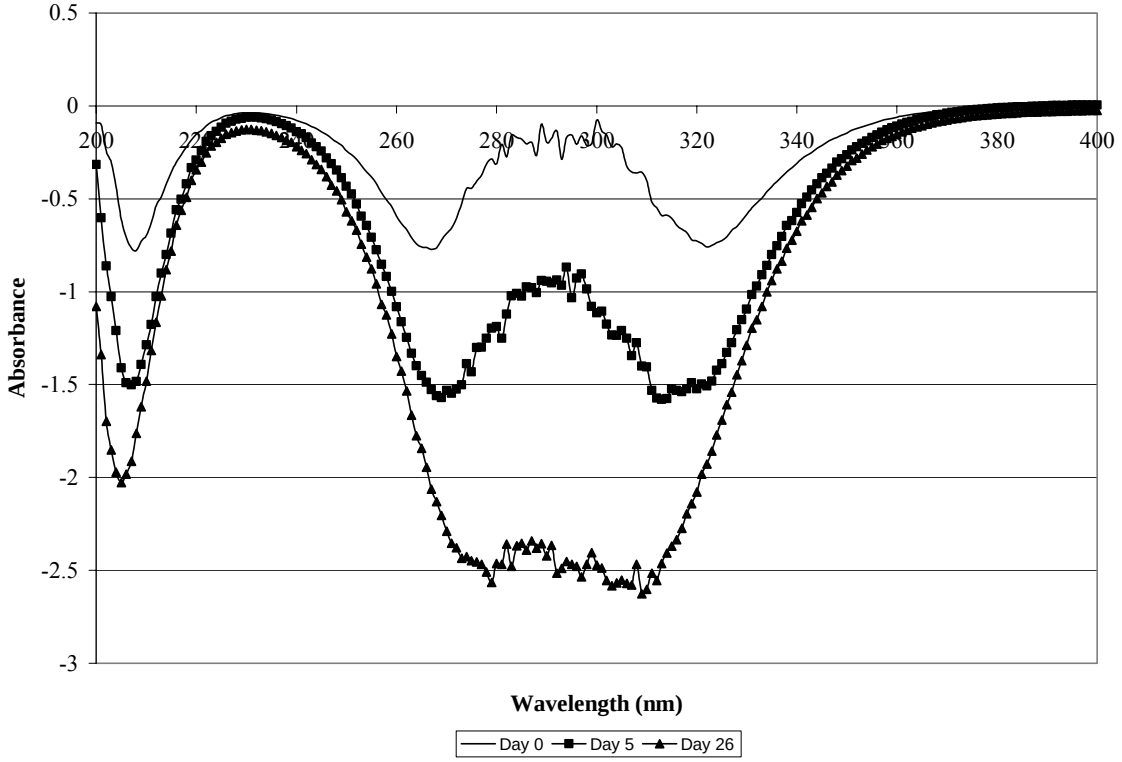


Figure 23. Spectral scans of a biocidal complex solution against a sodium hypochlorite baseline. A biocidal complex solution (hydrogen peroxide: sodium hypochlorite, pH 11) at a 1:10 ratio (2,500 ppm H_2O_2) was stored in the presence of U/V light at 24˚C for a 26 day period. Differential scans (200 nm to 400 nm) of the solution were taken on day 0, 5 and 26 against a sodium hypochlorite (25,000 ppm) baseline.

Table 10. Stability of biocidal complex, sodium hypochlorite and hydrogen peroxide at different pH, temperature and U/V light conditions.

Treatment*		Half-life (days)			
	pH	4˚C	24˚C	37˚C	24˚C U/V light
Hydrogen peroxide	7	252	196	122	78
Hydrogen peroxide	6.5	187	68	21	75
Sodium hypochlorite	10.9	1386	336	319	87
Sodium hypochlorite	6.5	30	22	20	18
Biocidal Complex	11	204	140	135	33
Biocidal Complex	6.5	41	45	22	16

*= Final concentrations tested were 2,500 ppm hydrogen peroxide and/or 2,5000 ppm sodium hypochlorite.

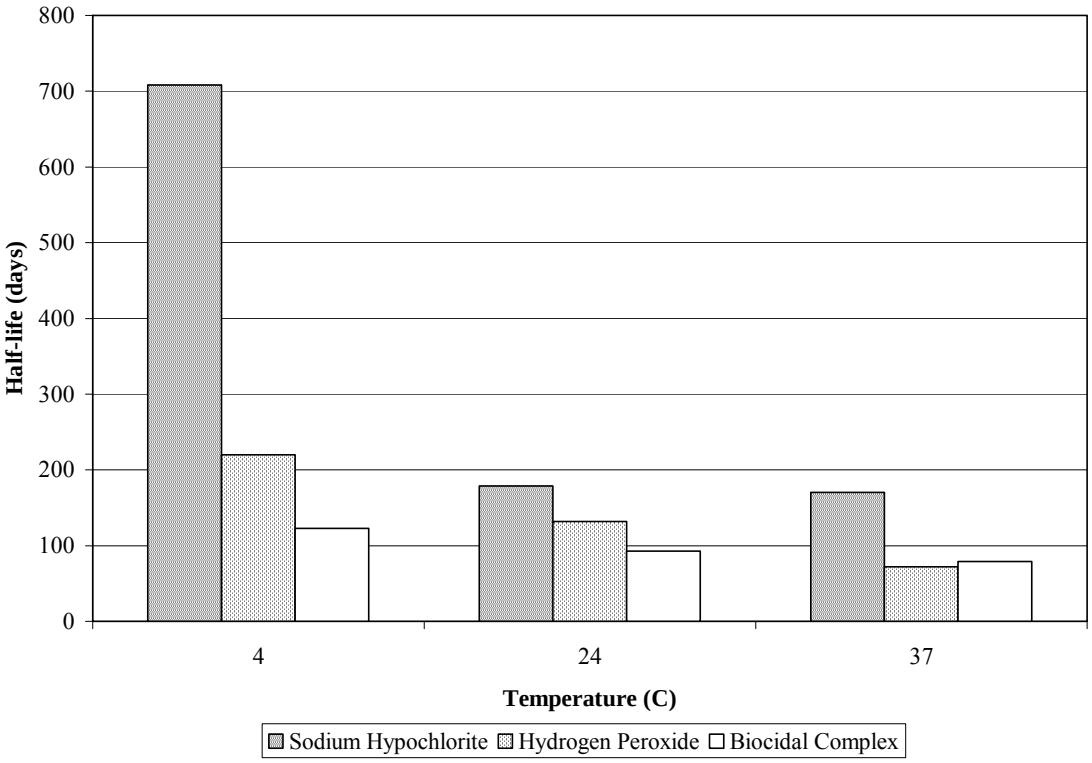


Figure 24. Temperature effect on biocide stability. Biocidal complex, sodium hypochlorite and hydrogen peroxide solutions were stored in the dark at 4˚C, 24˚C and 37˚C for 42 days. The stability of each solution was monitored by conducting differential wave scans (200 nm to 450 nm) on each sample and calculating the change in area of each scan over a 42 day period. Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at a ratio of 1:10 (2,500 ppm H_2O_2), sodium hypochlorite at 25,000 ppm and hydrogen peroxide at 2,500 ppm.

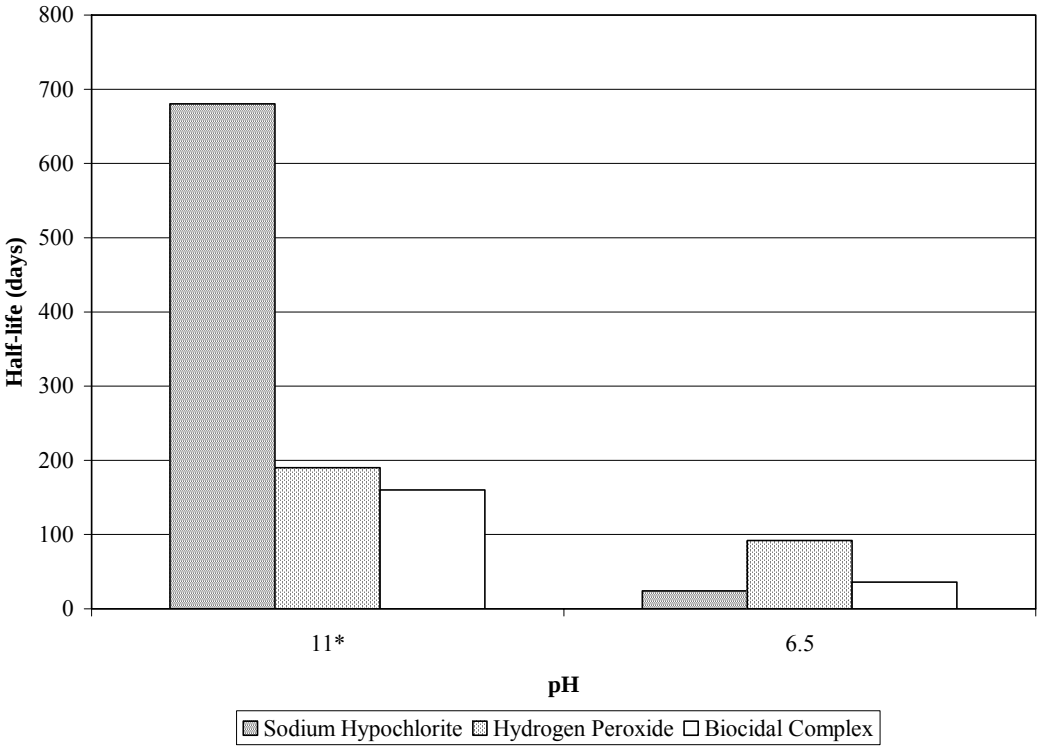


Figure 25. pH effect on biocide stability. The pH of biocidal complex, sodium hypochlorite and hydrogen peroxide solutions was adjusted from 11(*****or pH 7 for hydrogen peroxide) to 6.5 with concentrated HCl. The stability of each solution was monitored by conducting differential wave scans (200 nm to 450 nm) on each sample and calculating the change in area of each scan over a 42 day period. Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at a ratio of 1:10 (2,500 ppm H_2O_2), sodium hypochlorite at 25,000 ppm and hydrogen peroxide at 2,500 ppm.

hydrogen peroxide (pH 7) and biocidal complex (pH 11) were 680 days, 190 days and 160 days, respectively. Adjusting the pH of the biocidal complex from 11 to 6.5 reduced its stability by 78% (124 days). The half-life of sodium hypochlorite dropped 97% (656 days), and the stability of hydrogen peroxide was reduced by 52% (98 days).

Exposure of test solutions to U/V light reduced their stability. The half-lives of sodium hypochlorite, hydrogen peroxide and biocidal complex solutions stored in the dark were 179 days, 132 days and 93 days, respectively (Figure 26). A drop in stability of 73% (68 days) was observed for biocidal complex solutions upon exposure to U/V light. Sodium hypochlorite decreased by 70% (126 days) and hydrogen peroxide by 42% (55 days). Overall, the half-life of biocidal complex was affected by heat, acidity and light.

3.3.3. Effect of Storage on Biocidal Complex Activity. Biocidal complex solutions (pH 11) were stored in the dark at 4˚C, 24˚C and 37˚C for 42 days. The activity of each test solution was tested against *B. subtilis* spore-contaminated stainless steel plates on day 0, 2, 4, 7 and 42 for a contact time of 15 min. The spore population on stainless steel plates averaged 6 log CFU cm^{-2}. Results are shown in Figure 27. The activity of biocidal complex against spores was stable from day 0 to day 7 with percent kills in the range of 79% to 100%. Spore reductions were in the range of 5 to 6 log CFU cm^{-2}. A 23% or 46% loss of activity was observed for test solutions stored at 24˚C or 37˚C for 42 days, with spore reductions of 4.8 or 3.4 log CFU cm^{-2}, respectively. No loss of activity was observed in test solutions stored at 4˚C.

3.3.4. Linear Relationship between Percent Kill and Area. Differential scans of a biocidal complex solution (pH 11), stored in the dark at 24˚C, were taken on day 0, 4, 7 and 42. The area below the curve of each biocidal complex scan (200 nm to 450 nm) was calculated and plotted against the percent kill data obtained after treating *B. subtilis* spore

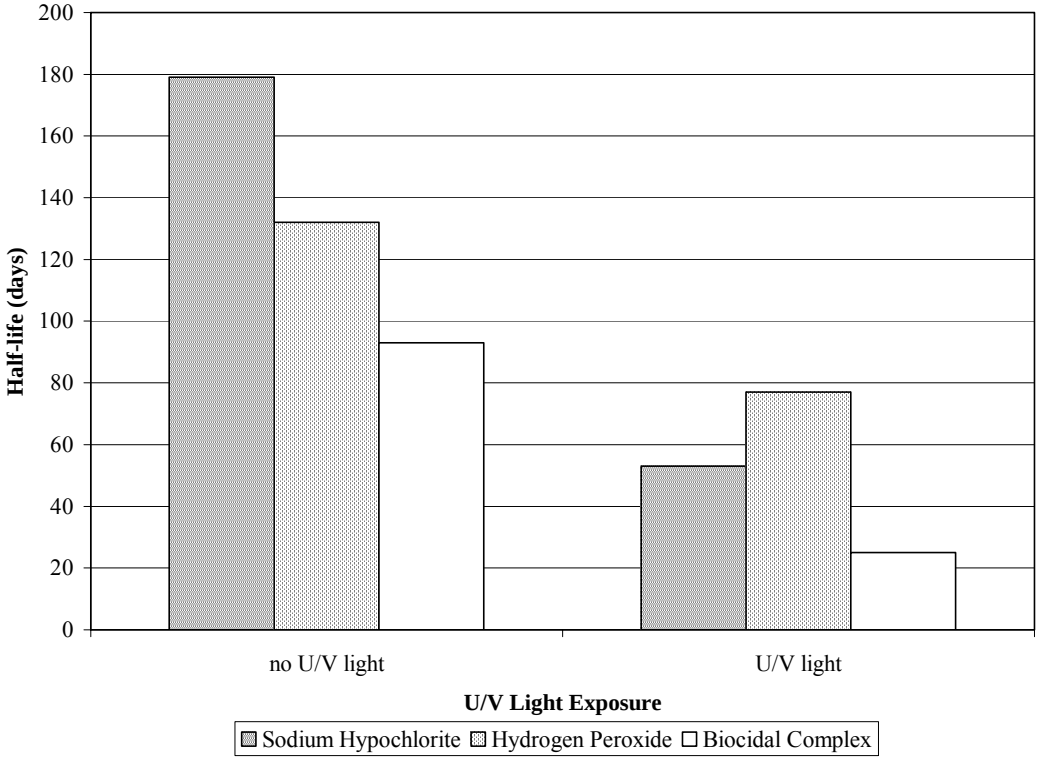


Figure 26. Effect of U/V light exposure on biocide stability. Biocidal complex, sodium hypochlorite and hydrogen peroxide solutions were stored in the presence or absence of U/V light at 24˚C for 42 days**.** The stability of each solution was monitored by conducting differential wave scans (200 nm to 450 nm) on each sample and calculating the change in area of each scan over a 42 day period. Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at a ratio of 1:10 (2,500 ppm H_2O_2), sodium hypochlorite at 25,000 ppm and hydrogen peroxide at 2,500 ppm.

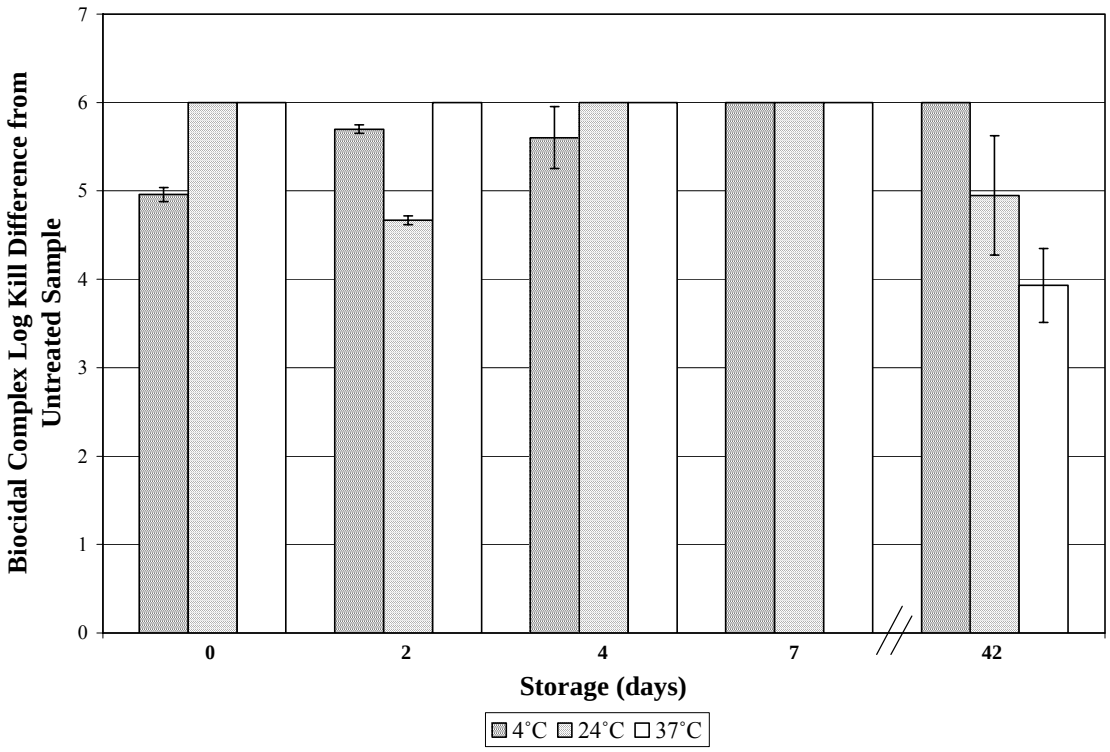


Figure 27. Effect of storage on biocidal complex activity against *B. subtilis* spore-contaminated stainless steel plates with a 15 min exposure. Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at a ratio of 1:10 (2,500 ppm H_2O_2). Test solutions were stored at 4˚C, 24˚C and 37˚C for 42 days. The antimicrobial activity of each test solution was analyzed on day 0, 2, 4, 7 and 42. Initial spore population on stainless steel plates prior to treatment with the biocide averaged 6 log CFU cm^{-2}. Error bars show the standard error of the mean.

covered stainless steel plates with the biocidal complex solution on day 0, 4, 7 and 42 for 5 min (Figure 28). The percent kill was directly proportional to the area below the curve for each biocidal complex wave scan. A 76% kill and an area of ∫Absorbance(λ)(d) λ= 279 were obtained on day 0. The percent kill of the biocidal complex decreased by 15% or 47% when stored for 7 days or 42 days at 24˚C, respectively. The area below the curve for each scan was reduced by 4% and 7%.

3.4. Singlet Oxygen

3.4.1 Production of Singlet Oxygen by the Biocidal Complex. Trans-1(2'- methoxyvinyl) pyrine (t-MVP) was used to detect the presence of singlet oxygen produced by the biocidal complex at a 1:10 ratio (2,500 ppm hydrogen peroxide). The fluorescent emission spectra from the biocide complex and each component were collected before and after triggering the reaction with *B. cereus* spores to a final concentration of 10^7 spores ml^{-1}. Figure 29 shows emission spectra for singlet oxygen from biocidal complex before and after *B. cereus* spore addition. Singlet oxygen emission, by strength of emission, in order of greatest to least, was hydrogen peroxide, biocidal complex and sodium hypochlorite. A 10 µl addition of a suspension of *B. cereus* spores produced a burst of emission in biocidal complex and hydrogen peroxide solutions. Singlet oxygen production from hydrogen peroxide showed a 72% increase after addition of spores, which was significantly ($P < 0.05$) different from biocidal complex (6%) or sodium hypochlorite (0%).

3.5. Response of *Pseudomonas aeruginosa* ATCC 19142 to Biocide Treatment

3.5.1. Effect of Attachment Status and Surface on Biocide Efficacy. Effectiveness of biocidal complex, hydrogen peroxide and sodium hypochlorite against six days old biofilms of *P.aeruginosa* on stainless steel or aluminum plates was tested at 1 min, 5 min and 20min exposure times. SEM images of six-day old *P. aeruginosa* biofilms growing on stainless steel

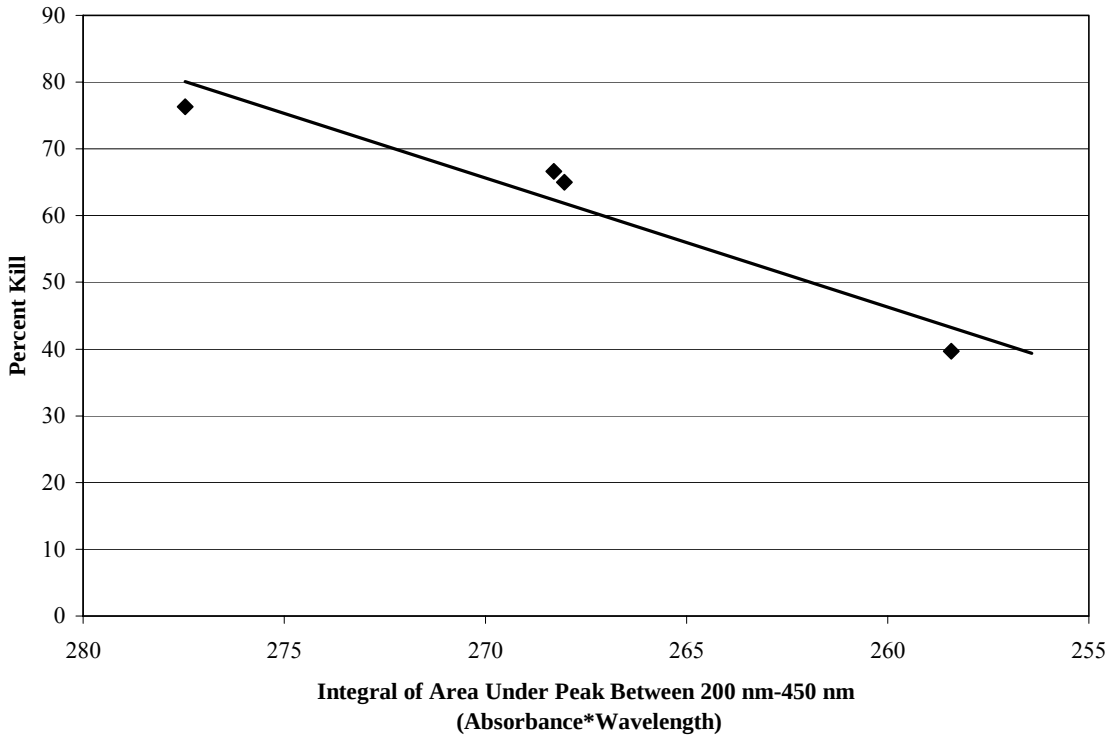


Figure 28. Relationship between percent kill and area. A biocidal complex solution (pH 11) was stored in the absence of U/V light at 24˚C for 42 days**.** The percent kill data was obtained by treating *B. subtilis* spore-covered stainless steel plates with the biocidal complex solution on day 0, 4, 7 and 42 for 5 min The stability of the solution was monitored by conducting differential wave scans (200 nm to 450 nm) on the sample and calculating the change in area of each scan on day 0, 4, 7 and 42. Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at a ratio of 1:10 (2,500 ppm H_2O_2).

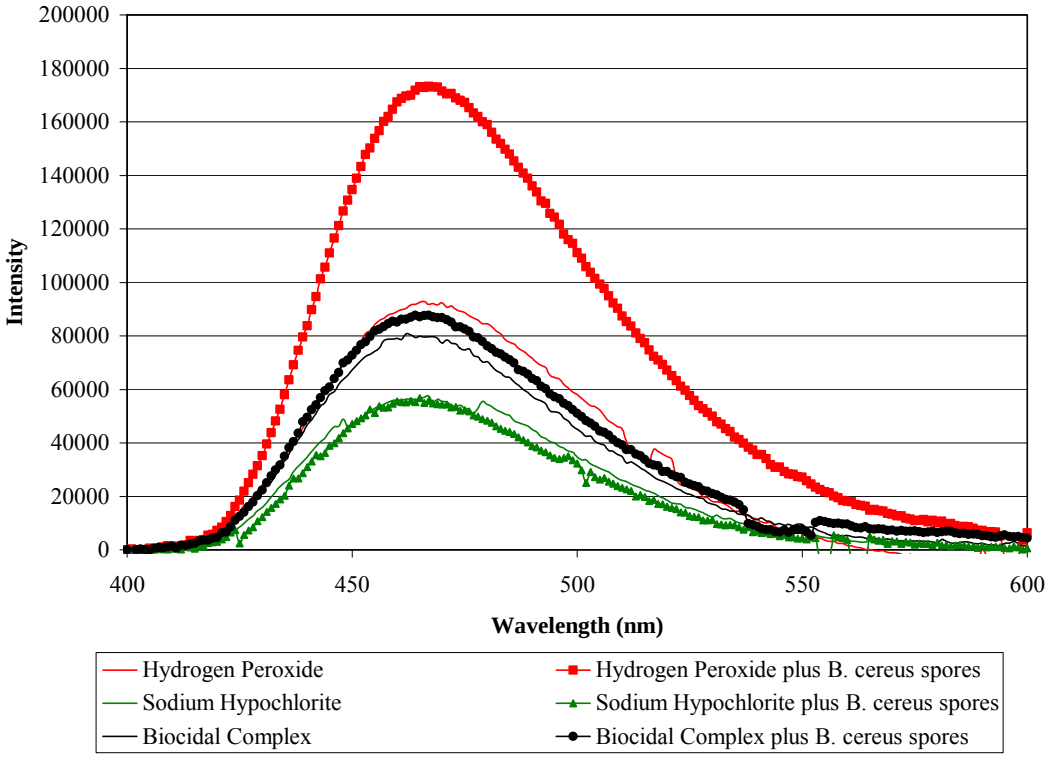
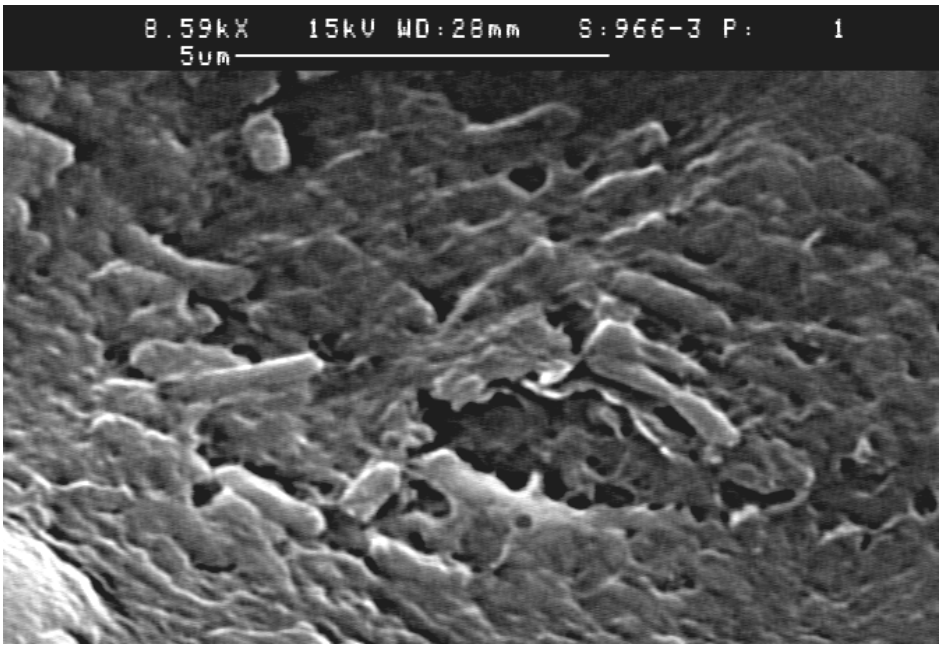


Figure 29. Singlet oxygen detection in a biocidal complex reaction. The biocidal complex reaction (hydrogen peroxide: sodium hypochlorite) was tested at a 1:10 ratio (2,500 ppm H_2O_2). Hydrogen peroxide or sodium hypochlorite, as controls, were tested at 2,500 ppm or 25,000 ppm, respectively. Trans-1- (2'-methoxyvinyl) pyrene was used to detect singlet oxygen, with excitation and emission wavelengths of 366 nm and 381-600 nm, respectively . The reaction was triggered with *B. cereus* spores to a final concentration of 10^7 spores ml^{-1}.

(Figure 30A) and aluminum (Figure 30B) plates revealed that cells were embedded within a layer of EPS. This EPS layer was thicker on the aluminum than on the stainless steel surfaces. *P. aeruginosa* on stainless steel or aluminum plates averaged 8.4 log in^{-2} or 8.7 log in^{-2} (Figures 31 and 32). There were significant ($P < 0.05$) differences in bacterial survival on stainless steel (7.9 log) and aluminum (8.4 log) plates after treatment. The effect of exposure time was also statistically ($P < 0.05$) significant for each metal surface. Survival means of 7.6, 6.4 and 5.7 log CFU in^{-2} were observed for aluminum samples after 1 min, 5 min and 20 min contact. However, a significant ($P<0.05$) difference of the mean was only observed at 5 min (5.9 log) or 20 min (4.6 log) contact times for stainless steel samples. Biocidal complex was the most effective sanitizer against attached Pseudomonads on both surfaces. Table 11 summarizes the percent kill data for attached *P. aeruginosa* after treatment with each sanitizer. Numbers of cells were significantly reduced ($P < 0.05$) after 1 min exposure to biocidal complex, resulting in 3.7 log or 5.7 log reductions, where as treatments with sodium hypochlorite resulted in 2.1 log or 3.1 log reductions for aluminum or stainless steel plates, respectively. Numbers of attached *P. aeruginosa* cells were significantly ($P < 0.05$) reduced when compared to control and sodium hypochlorite treated samples for both surfaces. A 5 min contact time with biocidal complex reduced counts by 6.8 log and 6.3 log, respectively. However, sodium hypochlorite only produced log reductions of 2.9 and 2.3, which were significantly ($P < 0.05$) different from biocidal complex treated samples. No cells could be recovered after 20 min treatment with biocidal complex on both surfaces. Count reductions of 4.9 log and 4.8 log were observed for sodium hypochlorite treated samples in both aluminum and stainless steel, respectively. No significant ($P < 0.05$) reduction in numbers was observed for hydrogen peroxide.

A.



B.

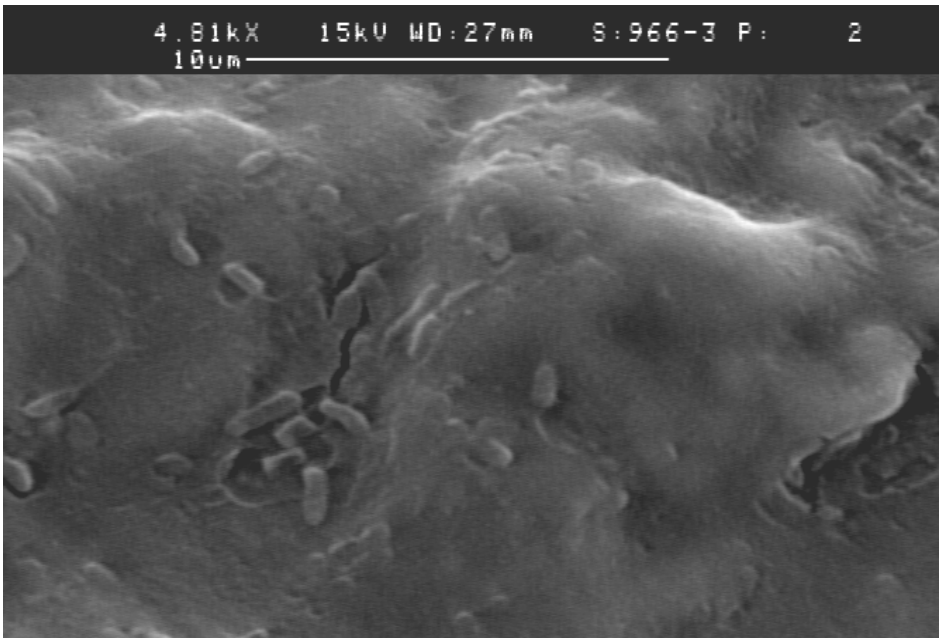


Figure 30. SEM images of *Pseudomonas aeruginosa* ATCC 19142 biofilms. Cells were grown in tryptic soy broth for six days and allowed to adhere to (a) stainless steel and (b) aluminum plates. The bars represent 5 μm at a 8.59 kX and 10 μm at a 4.81 kX magnification.

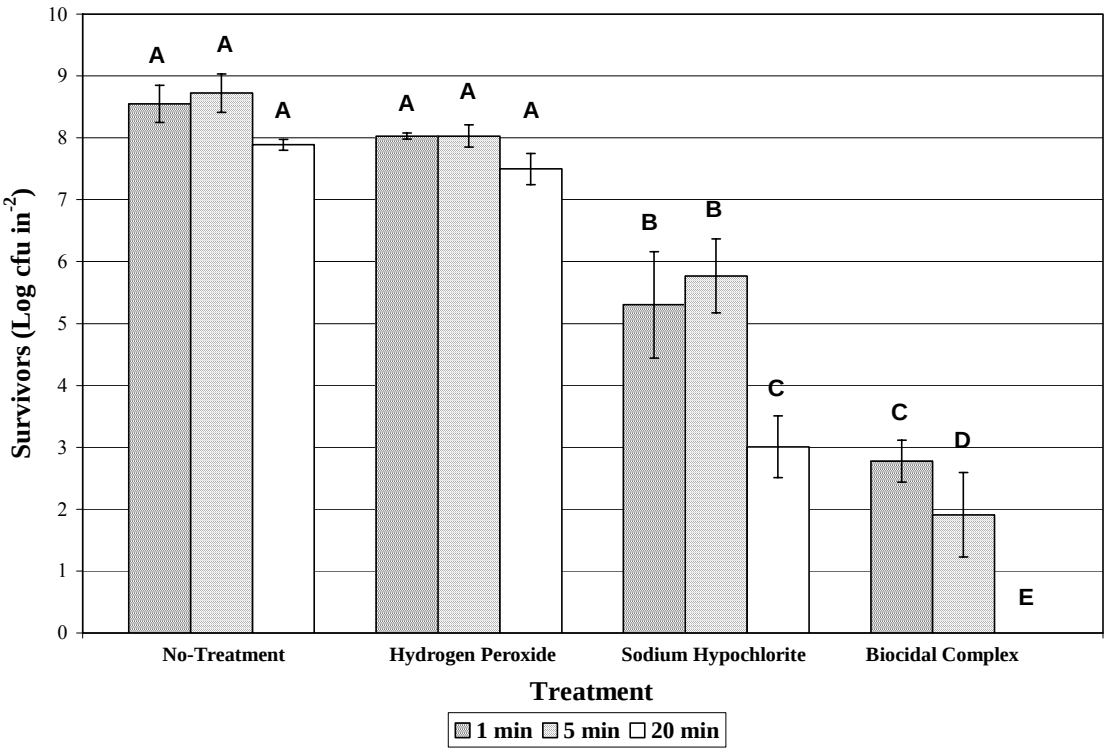


Figure 31. Biocide effectiveness against attached *Pseudomonas aeruginosa* ATCC 19142 on stainless steel plates. Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at a ratio of 1:10 (2,500 ppm H_2O_2) and hydrogen peroxide or sodium hypochlorite were used at 2,500 ppm or 25,000 ppm , respectively, for contact times of 1 min, 5 min and 20 min. Untreated samples were treated with sterile distilled water. Means with different superscripts indicate statistically significant differences ($P < 0.05$). Error bars show the standard error of the mean.

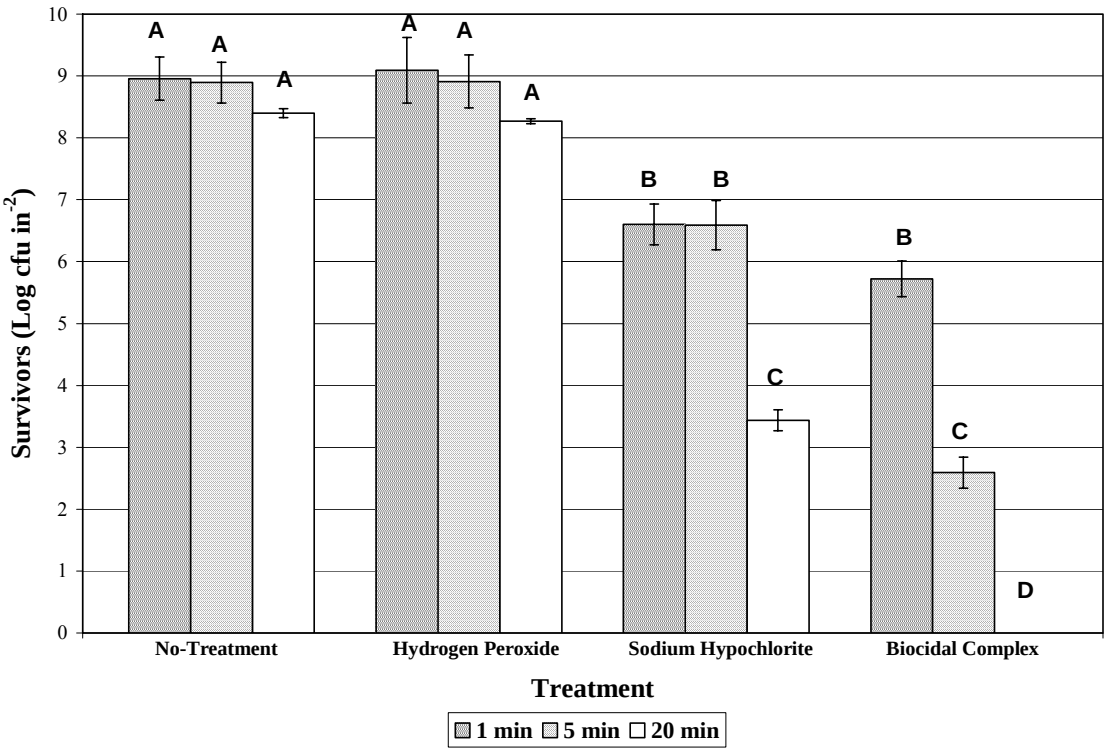


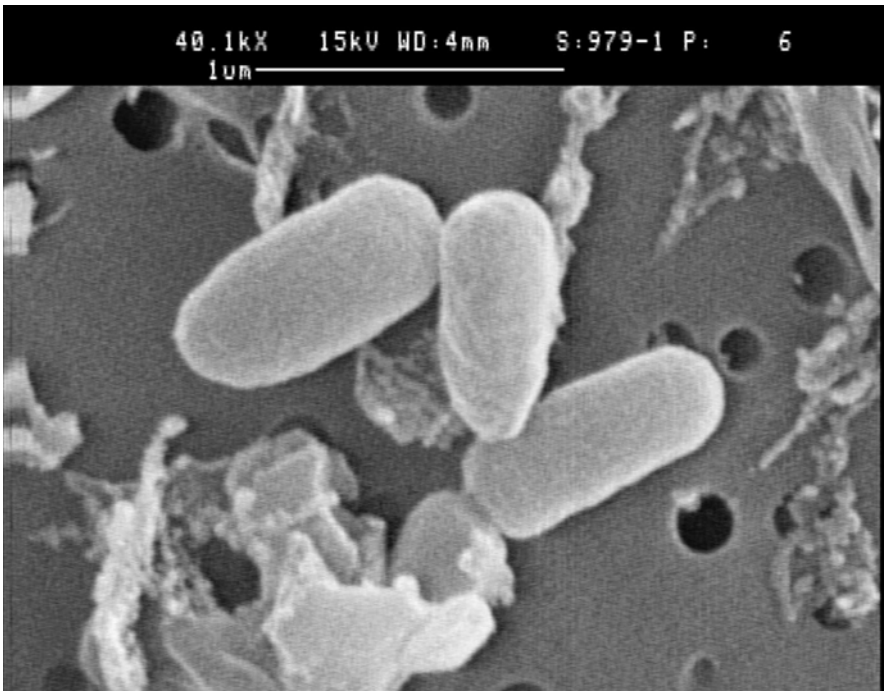
Figure 32. Biocide effectiveness against attached *Pseudomonas aeruginosa* ATCC 19142 on aluminum plates. Biocidal complex (hydrogen peroxide: sodium hypochlorite) was tested at a ratio of 1:10 (2,500 ppm H_2O_2) and hydrogen peroxide or sodium hypochlorite were used at 2,500 ppm or 25,000 ppm , respectively, for contact times of 1 min, 5 min and 20 min. Untreated samples were treated with sterile distilled water. Means with different superscripts indicate statistically significant differences ($P < 0.05$). Error bars show the standard error of the mean.

Table 11. Percentage kill data for attached *P. aeruginosa* after exposure to biocides.

Contact Time (min)	Biocidal Complex	Sanitizer Sodium Hypochlorite	Hydrogen Peroxide
Stainless Steel			
1	99.999	99.9	70
5	99.9999	99.9	80
20	99.999999	99.999	59
Aluminum			
1	99.9	99.6	<1
5	99.9999	99.5	4
20	99.999999	99.9999	26

3.5.2. Microscopy observations. Scanning electron micrographs of untreated, six days old, *P. aeruginosa* biofilms on aluminum surfaces showed rods of approximately 0.74 μm in length with smooth surfaces and no visible malformations (Figure 33A). By contrast, surfaces of *P. aeruginosa* cells exhibited shape distortions, indentations and slight elongation after 1 min (Figure 33B) and 5 min (data not shown) exposure to biocidal complex. Cells exposed to the biocidal complex for 20 min showed severe indentations, distortions and roughness of surfaces (Figure 34A). TEM images of 20 min treated cells revealed massive perforations to cell walls and cell membranes (Figure 34B). The average increase in length of *P. aeruginosa* cells on aluminum surfaces did not change significantly ($P < 0.05$) after 5 min treatment with biocidal complex. However, a significant ($P < 0.05$) increase in cell length from 0. 74 μm to 1.44 μm was observed after 20 min treatment with biocidal complex (Figure 35). *P. aeruginosa* attached to stainless steel plates revealed significant ($P < 0.05$) cell length increments after 1 min treatment. The average length increased from 0.8 μm before treatment, to 0.96 μm after 1 min and then to 1.02 μm and 1.5 μm after 5 min and 20 min treatments (Figure 36). Previous reports have shown that an increase in cell length after sanitizer treatment may be an indication of cell injury (Lindsay and von Holy, 1999). Under confocal scanning electron microscopy (CSLM), attached *P. aeruginosa* cells appeared spread over the stainless steel and aluminum surfaces, forming loosely packed microcolonies. However, SEM observations through the depth of the biofilm between the upper and lower layers of the polysaccharide matrix revealed tightly packed bacterial cells within the biofilm's internal arrangement (Figures 37A and 37B). CSLM images revealed that all cells from stainless steel (Figure 38A) and aluminum surfaces (Figure 39A) were viable before treatment with biocidal complex. After sanitizer treatment for 1 min (Figure 38B and 39B), 5 min (Figures 38C and 39C) or 20 min (Figures 38D and 39D), cells

A.



B.

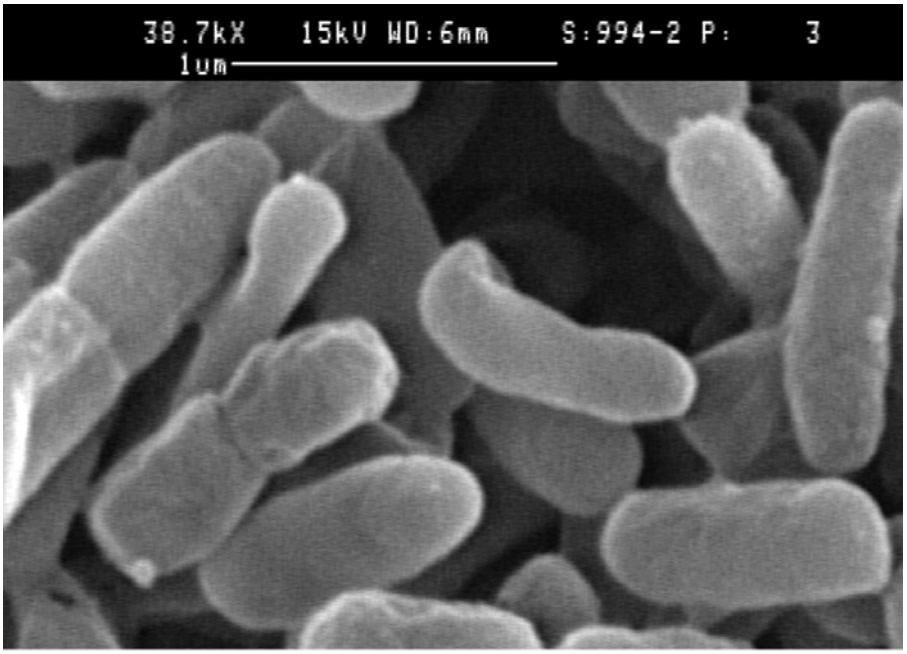
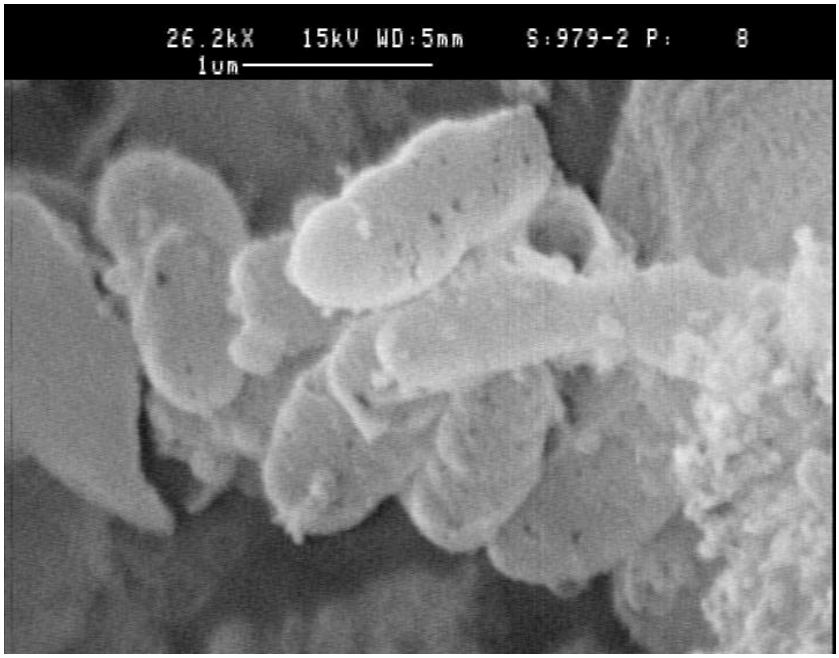


Figure 33. SEM images of *P. aeruginosa* cells from six days old biofilm formed on an aluminum plate. *Pseudomonas aeruginosa* cells (a) before treatment and (b) after 1 min treatment with biocidal complex (hydrogen peroxide: sodium hypochlorite) at a 1/10 ratio (2,500 ppm H_2O_2). Bars represent 1 μm at 40.1 kX and 38.7 kX magnification.

A.



B.

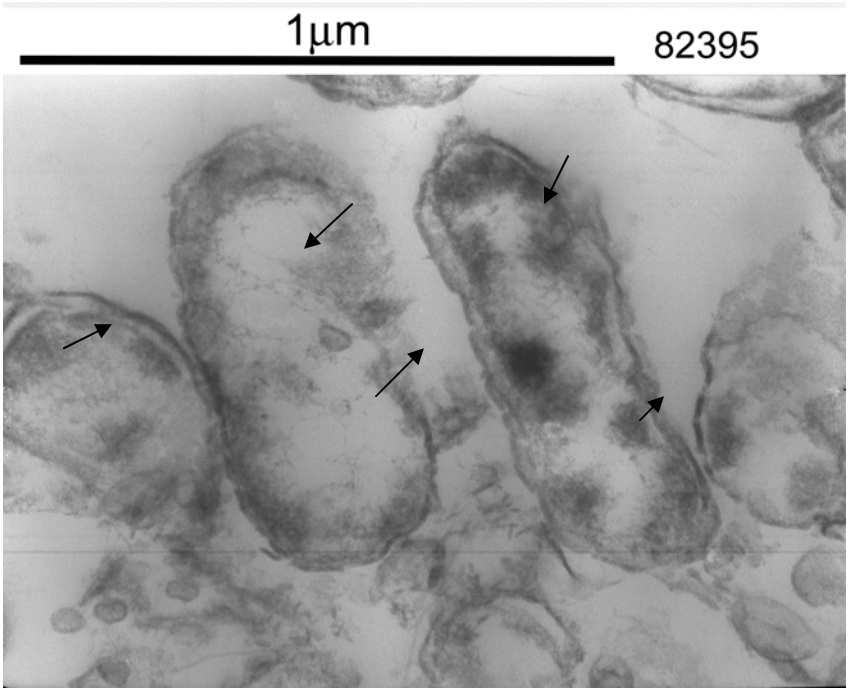


Figure 34. SEM and TEM images of *P. aeruginosa* cells from six days old biofilm formed on an aluminum plate. (a) SEM and (b) TEM images of cells after 20 min treatment with biocidal complex (hydrogen peroxide: sodium hypochlorite) at a 1/10 ratio (2,500 ppm H_2O_2). Arrows point at perforations within the cell wall and cell membrane. The bar represents 1 µm at 26.2 kX magnification.

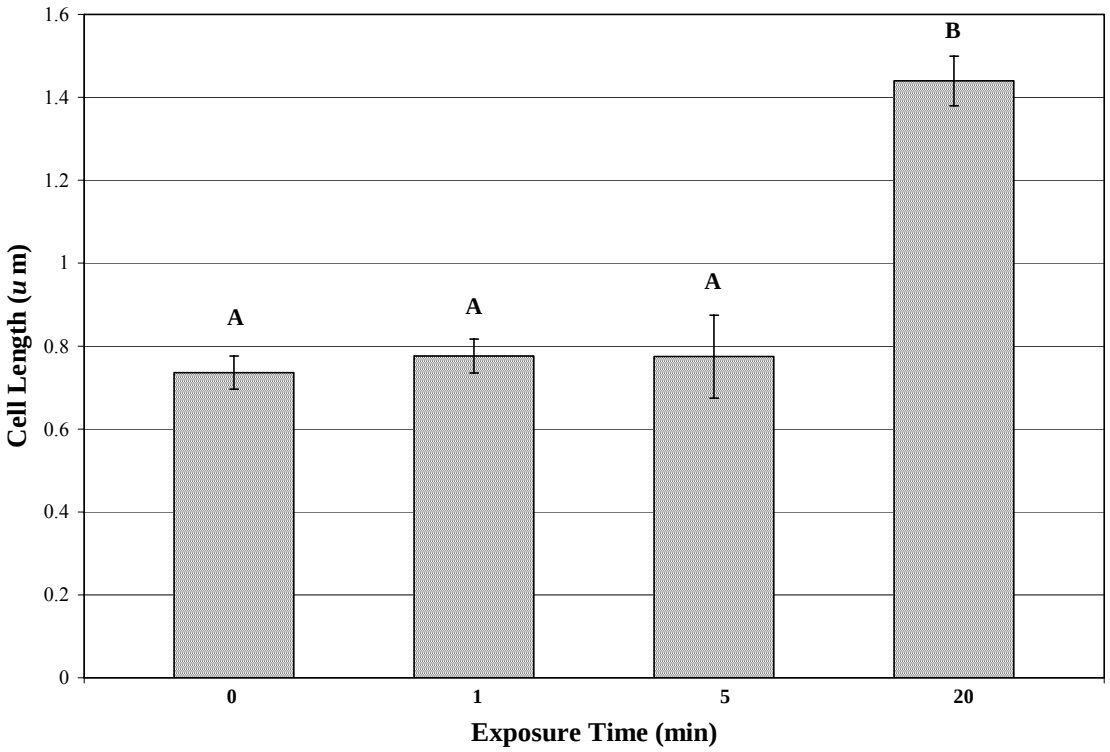


Figure 35. Average cell length estimates from SEM images of six days old *P. aeruginosa* ATCC 19142 biofilm on aluminum plates. Biofilm was grown on aluminum and treated with biocidal complex (hydrogen peroxide: sodium hypochlorite) at a 1/10 ratio (2,500 ppm H_2O_2) for 1 min, 5 min and 20 min. Control samples (0 min) were rinsed once with sterile distilled water. Means with different superscripts indicate statistically significant differences ($P < 0.05$). Error bars show the standard error of the mean.

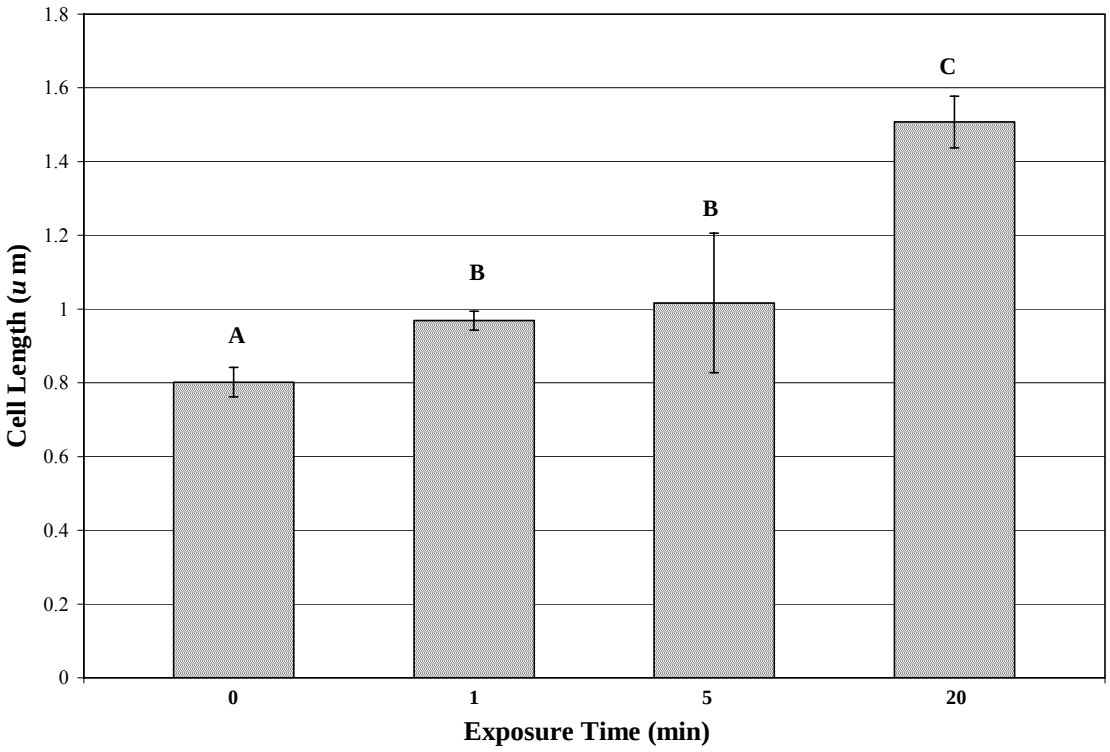
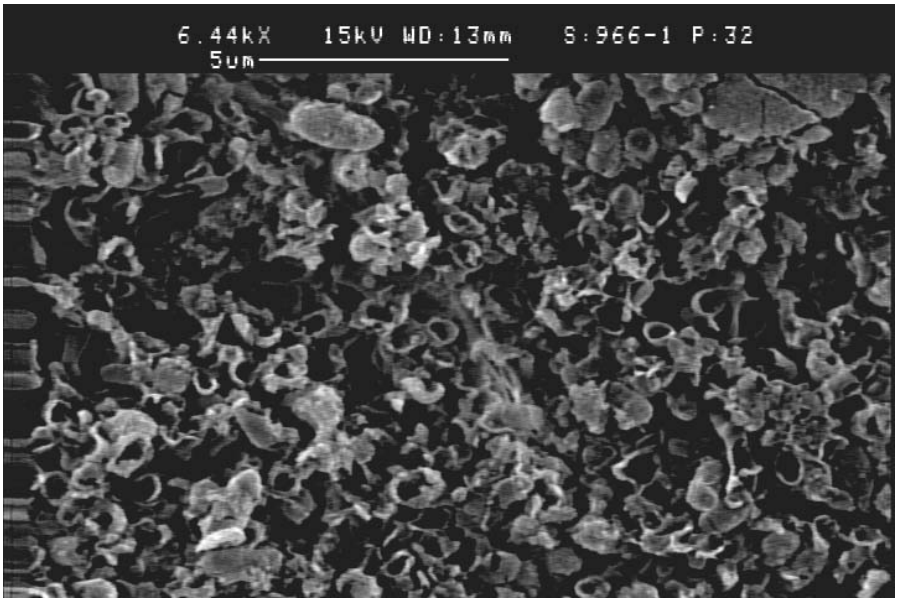


Figure 36. Average cell length estimates from SEM images of six days old *P. aeruginosa* ATCC 19142 biofilm on stainless steel plates. Biofilm was grown on stainless steel plates and treated with biocidal complex (hydrogen peroxide: sodium hypochlorite) at a 1/10 ratio (2,500 ppm H_2O_2) for 1 min, 5 min and 20 min. Control samples (0 min) were rinsed once with sterile distilled water. Means with different superscripts indicate statistically significant differences ($P < 0.05$). Error bars show the standard error of the mean.

A.



B.

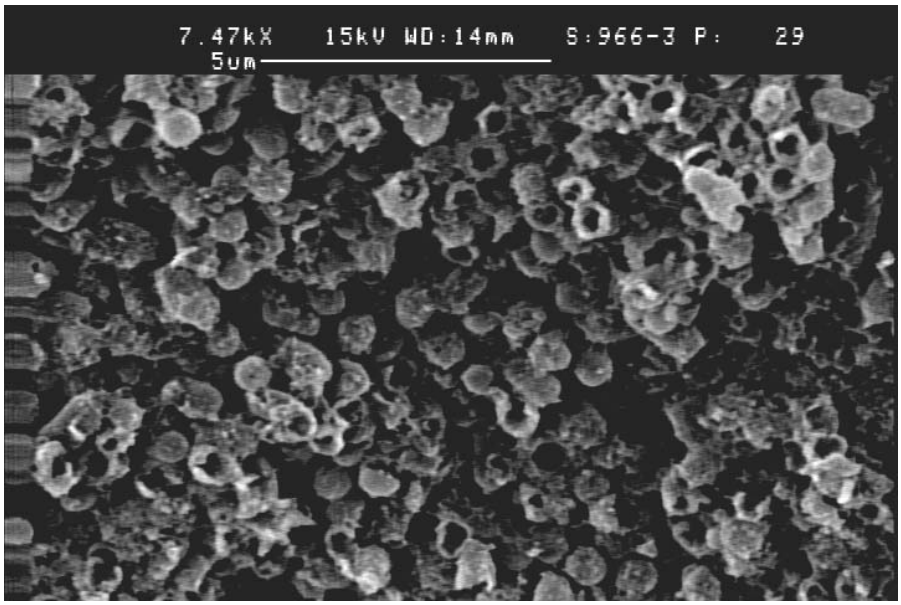


Figure 37. SEM images within the upper and lower layers of six days old *P. aeruginosa* biofilm. Tightly stack (a) stainless steel and (b) aluminum cells within the polysaccharide matrix. The bar represents 5 μm at 6.44 and 7.47 kX magnification.

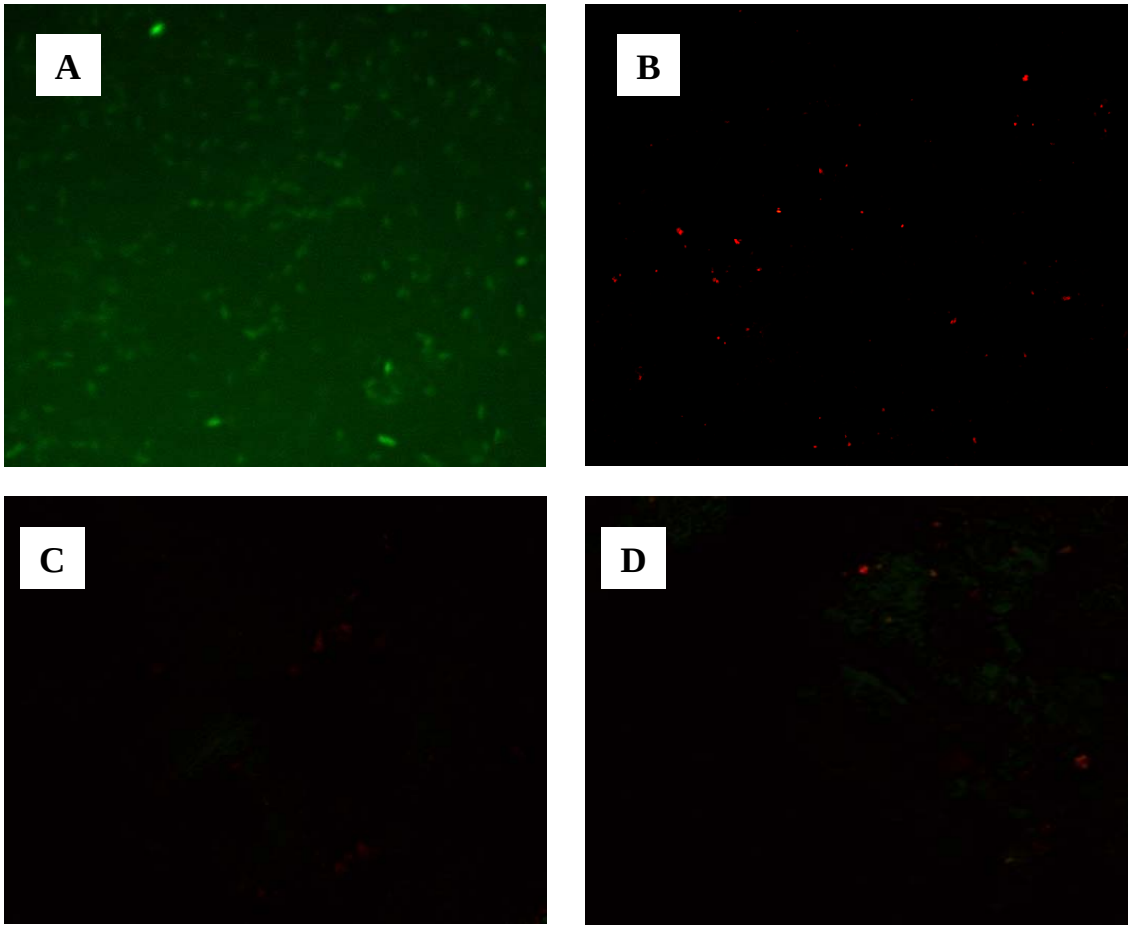


Figure 38. Confocal scanning laser images of six days old *P.aeruginosa* biofilm on stainless steel surfaces. Bacterial cells (a) before and after treatment with biocidal complex (hydrogen peroxide: sodium hypochlorite) at a 1/10 ratio (2,500 ppm H_2O_2)for (b) 1 min, (c) 5 min and (d) 20 min. Images taken at 40 X and 63 X magnification.

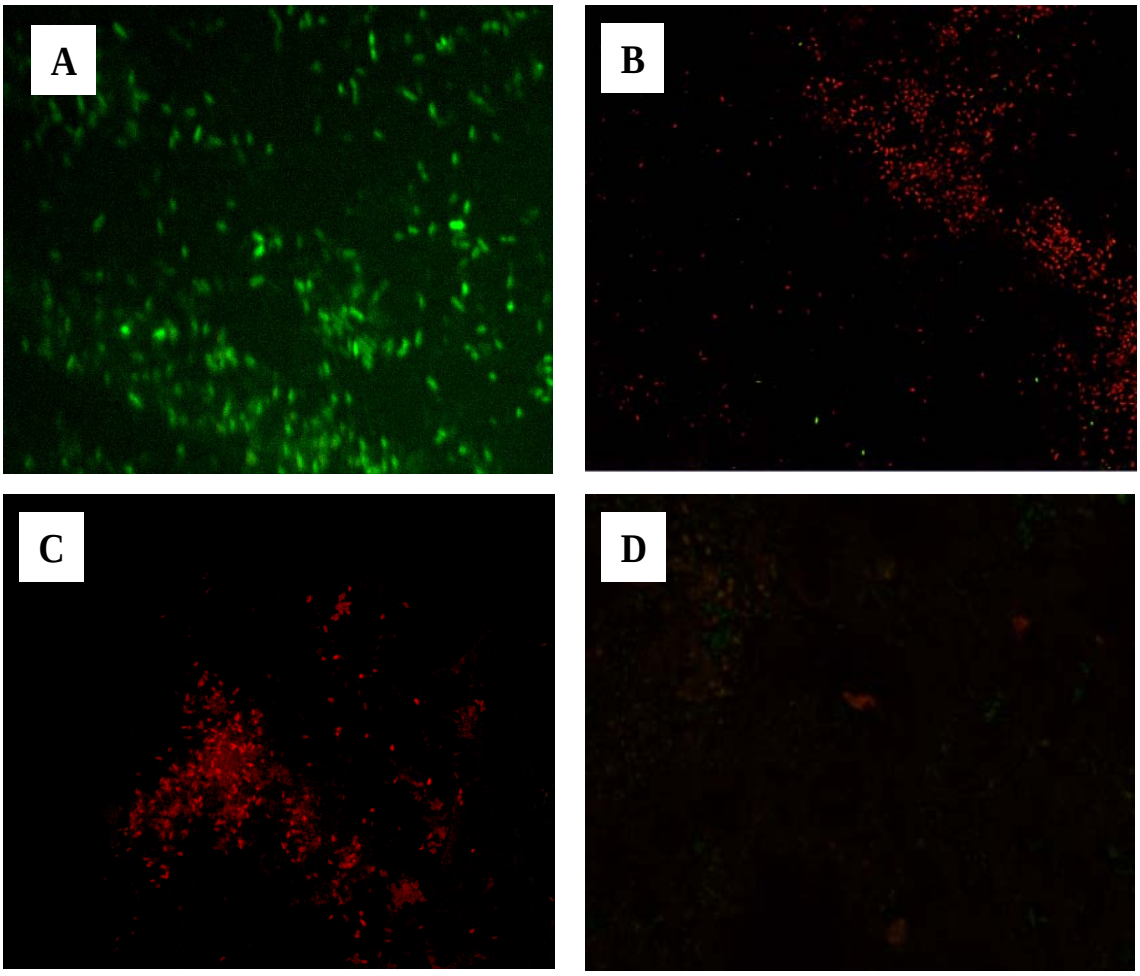


Figure 39. Confocal scanning laser images of six days old *P.aeruginosa* biofilm on aluminum surfaces. Bacterial cells (a) before and after treatment with biocidal complex (hydrogen peroxide: sodium hypochlorite) at a 1/10 ratio (2,500 ppm H_2O_2)for (b) 1 min, (c) 5 min and (d) 20 min. Images taken at 40 X and 63 X magnification.

stained red indicating cell injury. In addition to cell injury, a decrease in the number of stained (red) cells was observed on both surface materials after treatment for 1 min. A complete absence of stained cells from stainless steel surface plates was seen after 5 min treatment. However, the same effect was only evident after 20 min treatment of aluminum plates. An indication of the development of a thicker biofilm layer on aluminum over stainless steel surfaces

3.6. Fourier Transform Infrared Analysis of Biocide Treated *Pseudomonas aeruginosa* ATCC 19142 Biofilms

3.6.1. Cell Growth and Biofilm Formation on Aluminum and Stainless Steel Plates. *P. aeruginosa* biofilms, grown in TSB, were allowed to develop on stainless steel and aluminum surfaces over a period of 18 days. FTIR spectra of *Pseudomonas aeruginosa* biofilms on both surfaces revealed an array of bands of different intensities (Figure 41-Figure 48). No spectra were seen on the control plates (Figure 40). The control spectra lacked any of the bands that correspond to organic and/or inorganic material characteristic of biofilms. Individual FTIR spectrum of aluminum or stainless steel plates removed from batch cultures of *P. aeruginosa* on days 6, 12 or 18 are presented in Figures 41 – 43 and Figures 45 - 47, respectively. The combined spectra are shown in Figure 44 and Figure 48. The FTIR bands between 1800 $cm^{-1}$and 900 cm^{-1} correlate with spectra of proteins, polysaccharides and nucleic acids, macromolecules commonly found in biofilms. The protein region is characterized by the amide I (1652 cm^{-1}-1648 cm^{-1}) and amide II (1550 cm^{-1}-1548 cm^{-1}) bands. Prominent amide I bands (~ 1668 cm^{-1}-1640 cm^{-1}) and amide II bands (~ 1551 cm^{-1}-1538 cm^{-1}) were observed in biofilms attached to both aluminum and stainless steel surfaces. Amide I and amide II bands became more intense with time, an indication of protein accumulation over the 18 days of this experiment. Major increases in relative intensity of features near 1500 cm^{-1} and 1400 cm^{-1} appeared between day 6 (Figure 41) and day 18 (Figure 42). The band near 1500 cm^{-1} corresponds to the symmetric

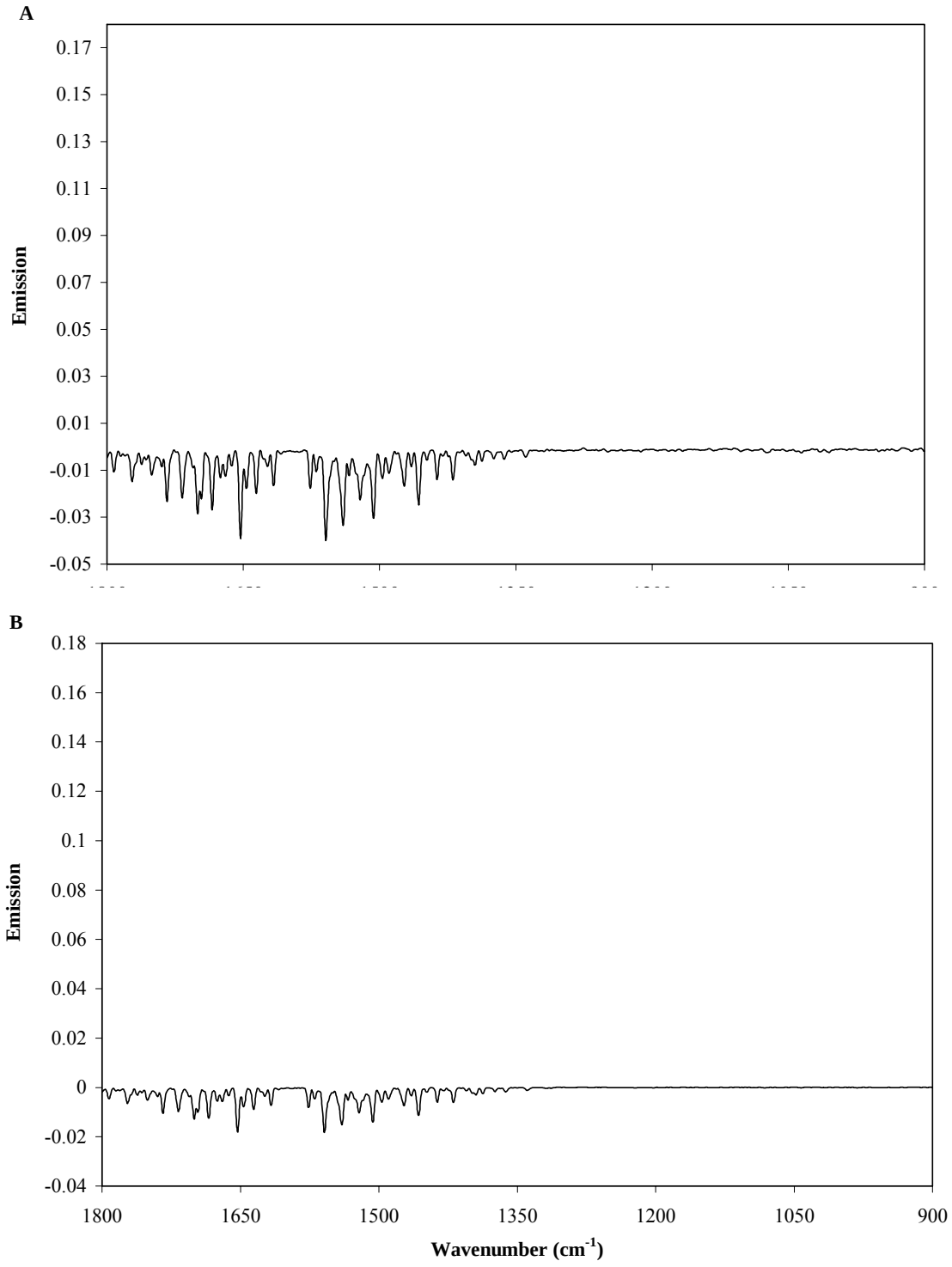


Figure 40. FTIR reflection spectra of an (a) aluminum and a (b) stainless steel plate before *P. aeruginosa* biofilm formation.

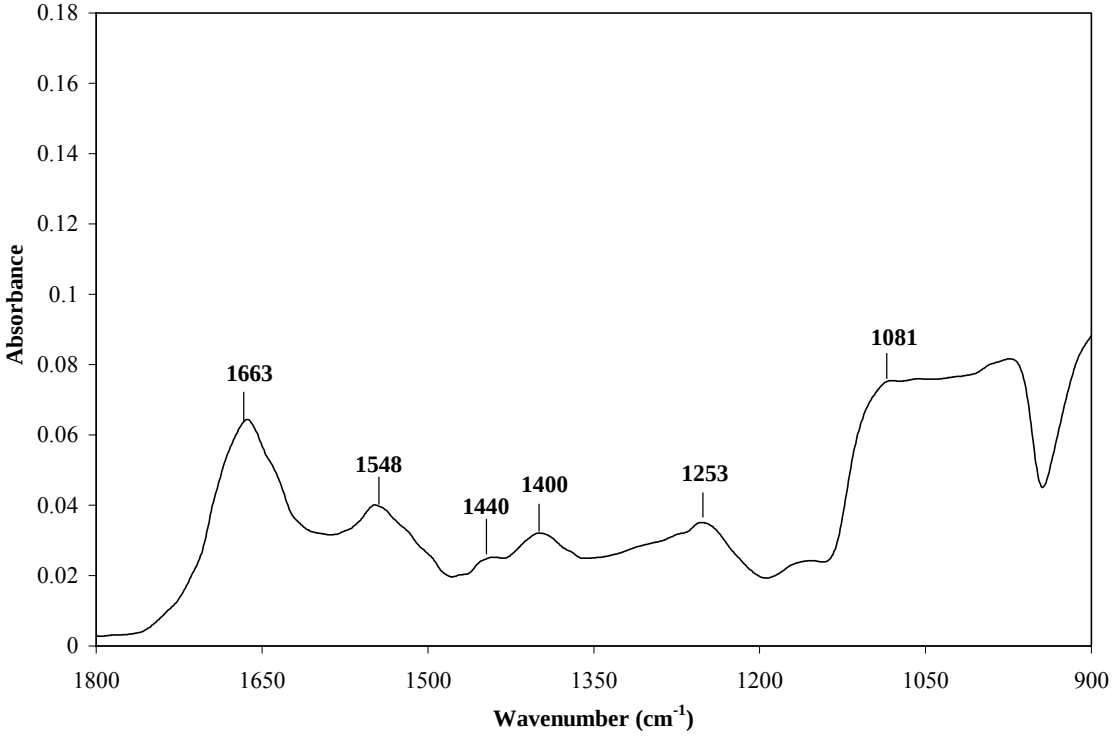


Figure 41. FTIR spectra of a six-day old *P. aeruginosa* biofilm on aluminum. *P. aeruginosa* cells were grown in TSB and allowed to adhere to aluminum plates for six days.

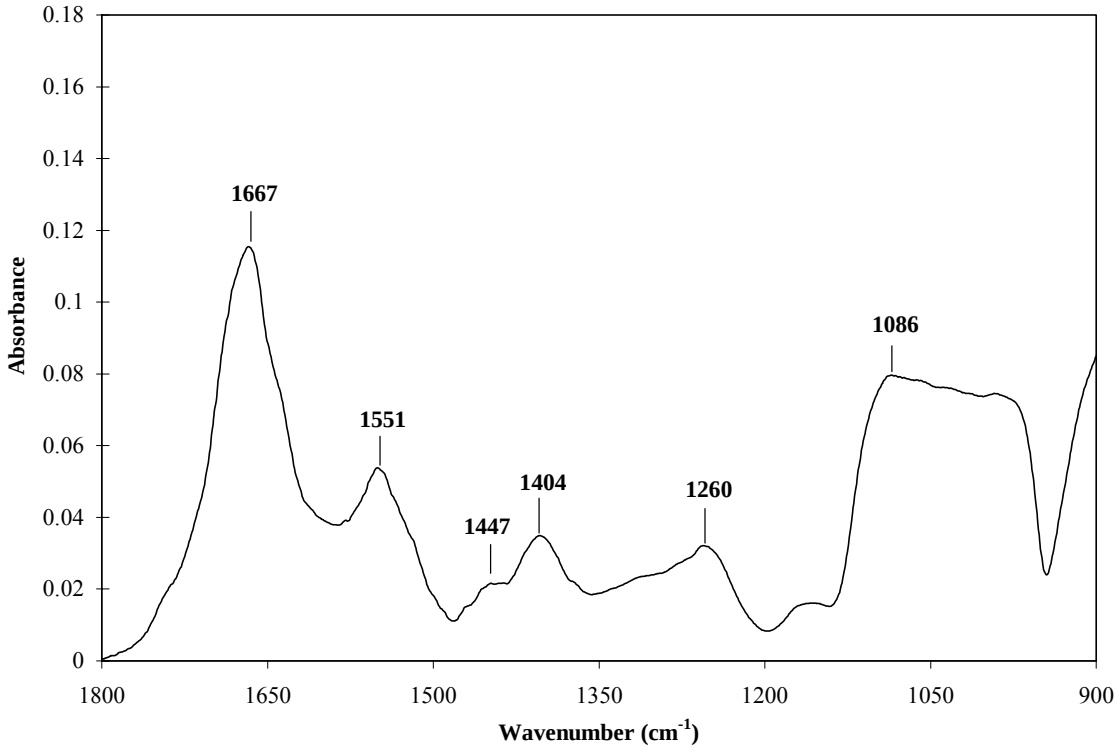


Figure 42. FTIR spectra of a twelve-day old *P. aeruginosa* biofilm on aluminum. *P. aeruginosa* cells were grown in TSB and allowed to adhere to aluminum plates for 12 days.

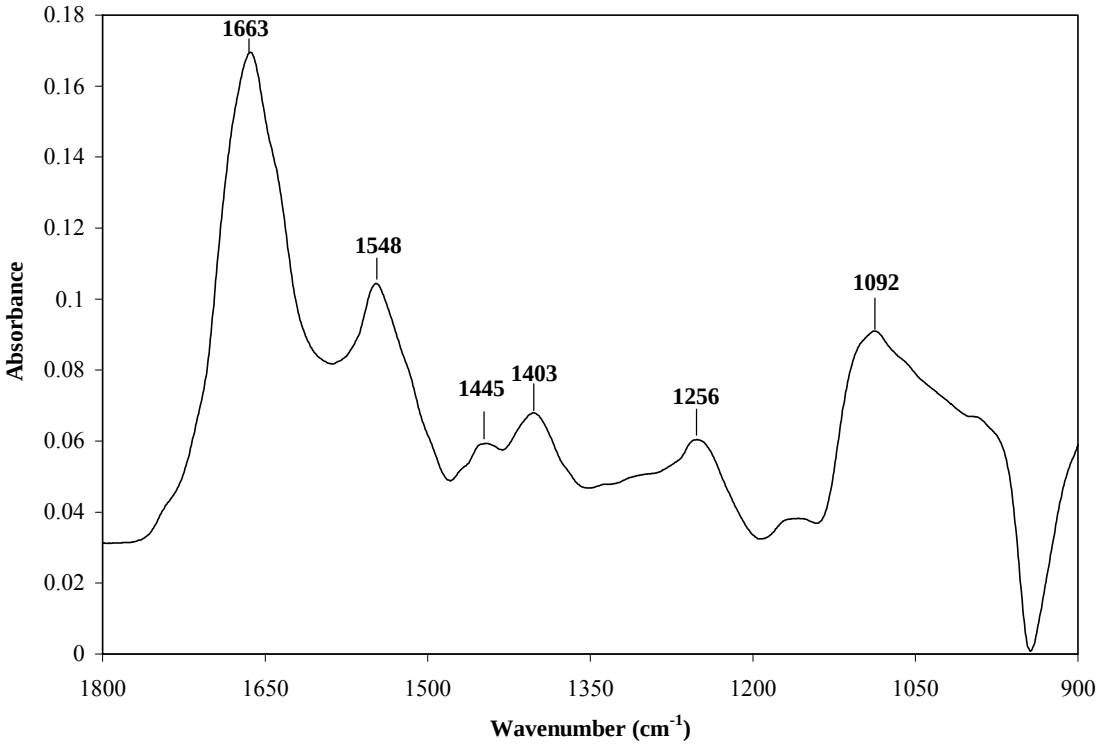


Figure 43. FTIR spectra of an eighteen-day old *P. aeruginosa* biofilm on aluminum. *P. aeruginosa* cells were grown in TSB and allowed to adhere to aluminum plates for 18 days.

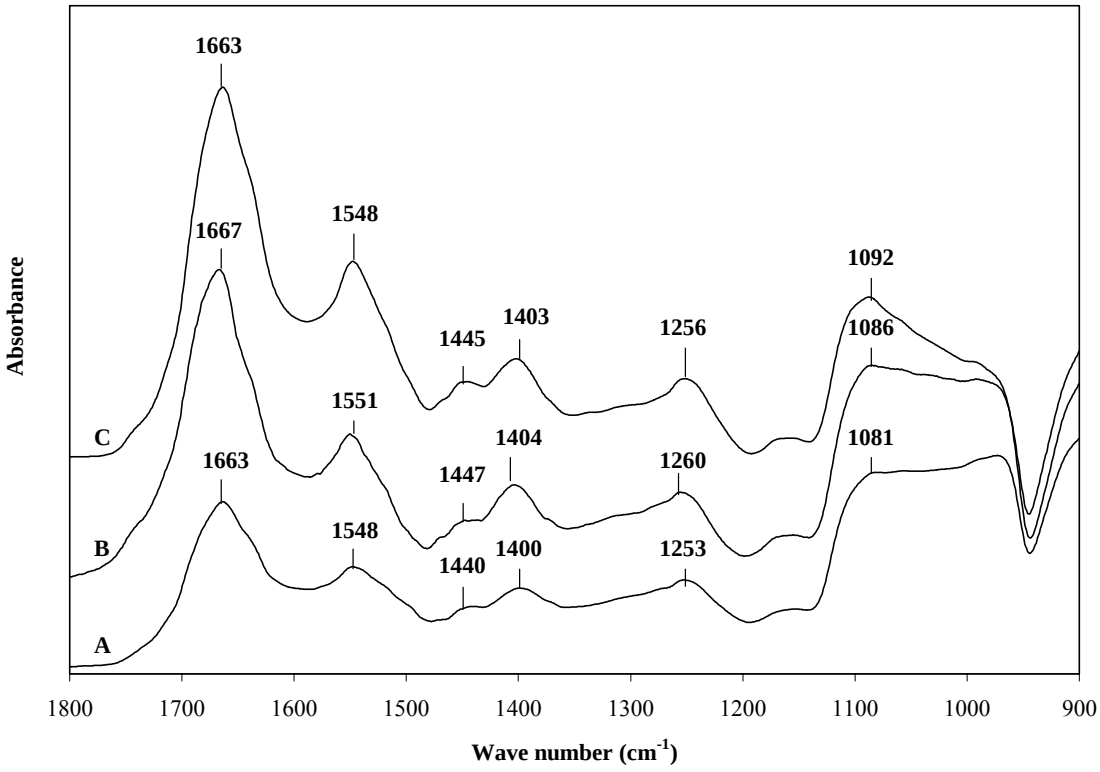


Figure 44. Combined FTIR spectra of *P. aeruginosa* biofilms on aluminum. *P. aeruginosa* cells were grown in TSB and allowed to adhere to aluminum plates over 18 days. Biofilm growth was monitored on (a) day 6, (b) day 12 and (c) day 18.

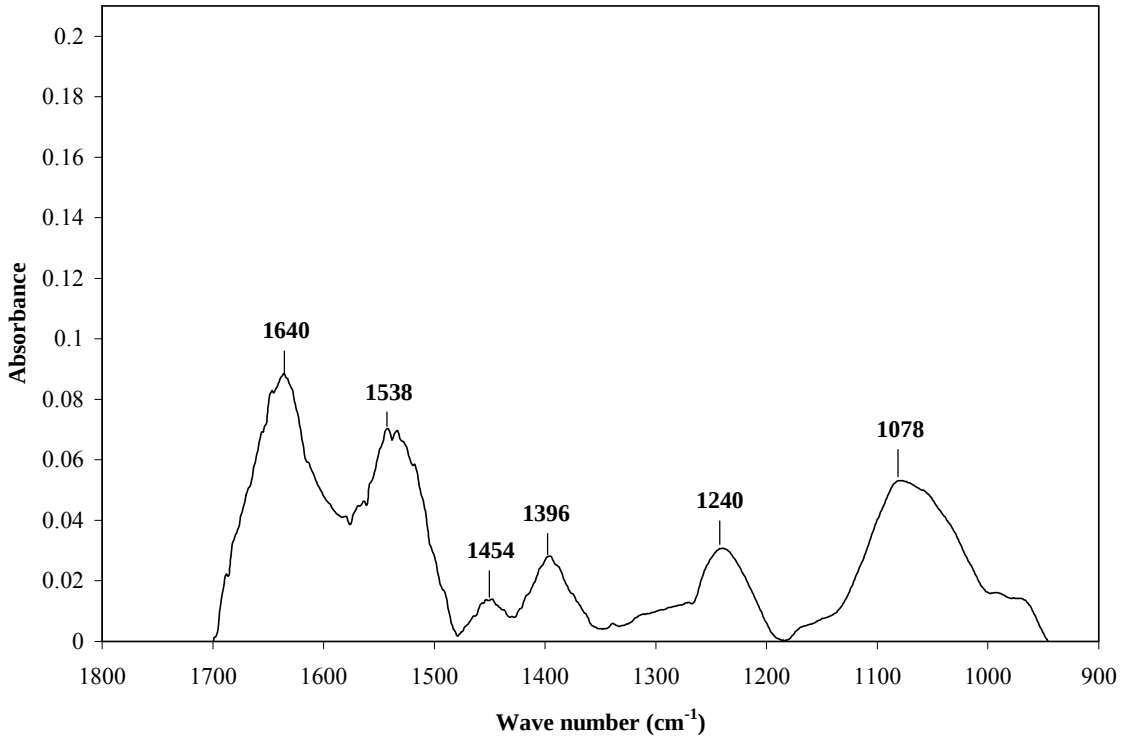


Figure 45. FTIR spectra of a six-day old *P. aeruginosa* biofilm on stainless steel. *P. aeruginosa* cells were grown in TSB and allowed to adhere to aluminum plates for 6 days.

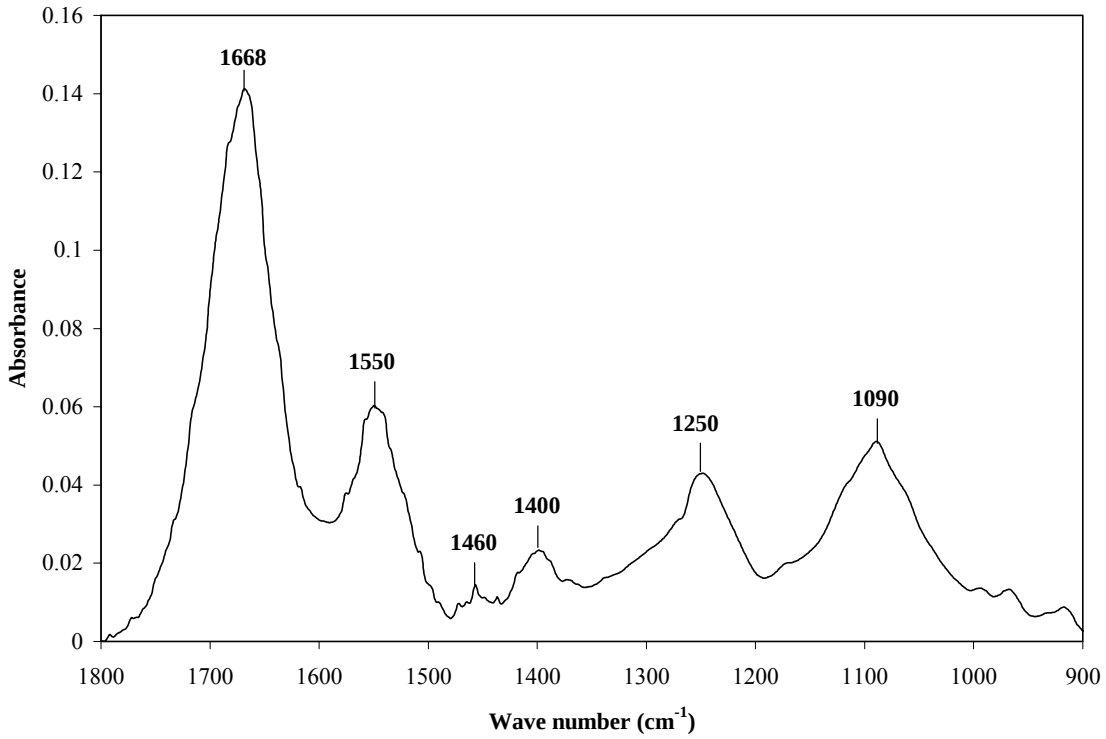


Figure 46. FTIR spectra of a twelve-day old *P. aeruginosa* biofilm on stainless steel. *P. aeruginosa* cells were grown in TSB and allowed to adhere to stainless steel plates for 12 days.

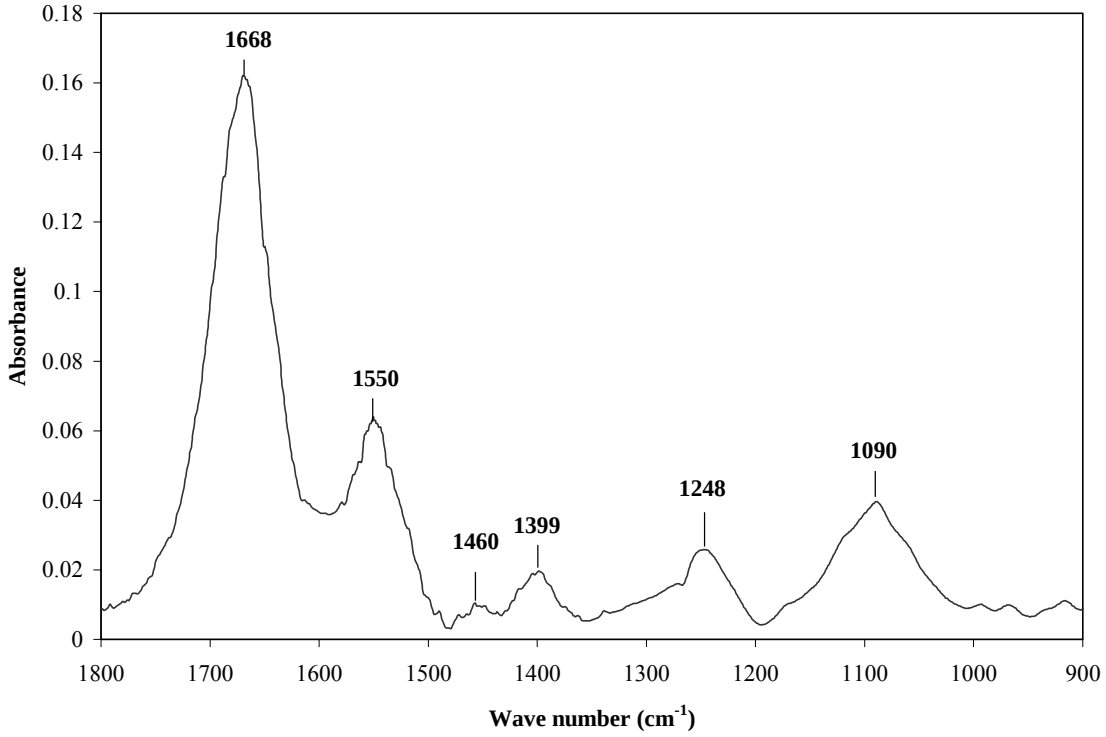


Figure 47. FTIR spectra of an eighteen-day old *P. aeruginosa* biofilm on stainless steel. *P. aeruginosa* cells were grown in TSB and allowed to adhere to stainless steel plates for 18 days.

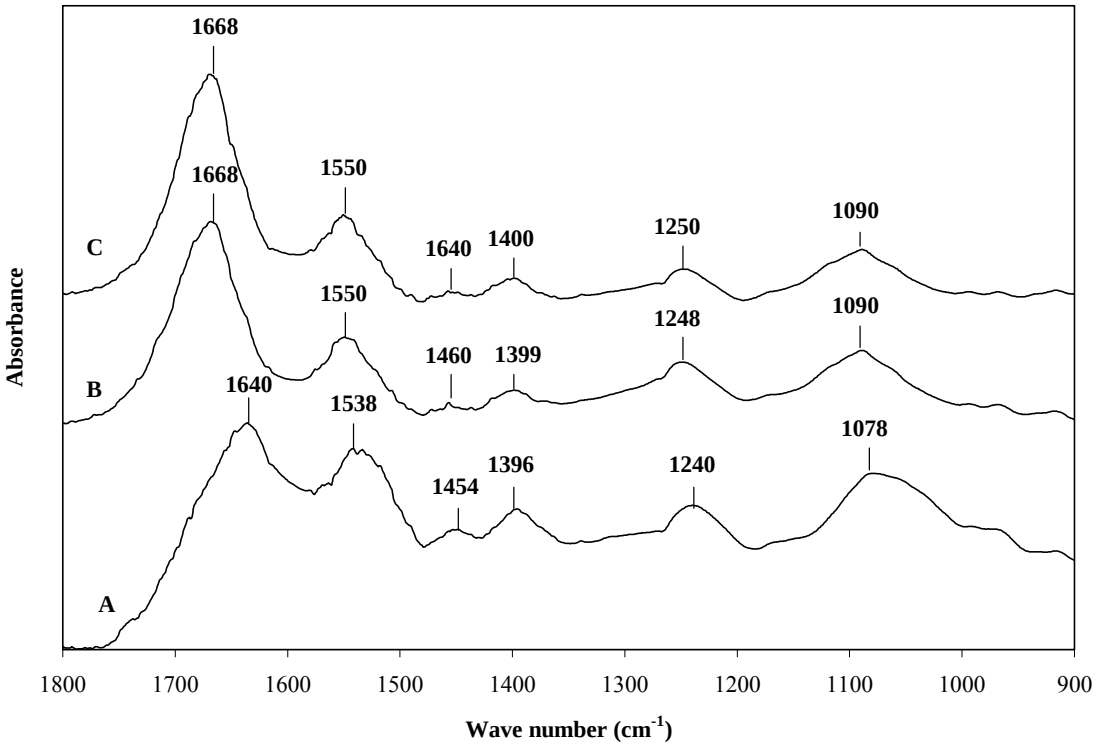


Figure 48. Combined FTIR spectra of *P. aeruginosa* biofilms on stainless steel. *P. aeruginosa* cells were grown in TSB and allowed to adhere to stainless steel plates over 18 days Biofilm growth was monitored on (a) day 6, (b) day 12 and (c) day 18.

deformation of a zwitterions adsorbed onto surfaces (Cheung et. al., 2000). The intensity of the band near 1400 cm^{-1} increased with time, corresponding to the symmetric stretch modes of a carboxylate group. This is an indication of an interaction between the $–COO^-$ group and the surface (Cheung et. al., 2000). Bands centered at 1081 cm^{-1} (Figure 44) and 1090 cm^{-1} (Figure 48) correspond to the C-O polysaccharide stretching mode (Bremer and Geesey, 1991). These bands became more intense by day 18, indicating the significant increase in polysaccharide concentration.

3.6.2. Biocidal Complex Removal of Biofilm from Stainless Steel and Aluminum Surfaces. *P. aeruginosa* biofilms formed on aluminum and stainless steel surfaces over 18 days were treated with biocidal complex for 5 min (Figure 49-Figure 54). Biofilm changes were monitored using FTIR analysis. Spectra of biofilm-treated aluminum plates are shown in Figure 49 (day 6), Figure 50 (day 12) and Figure 51 (day 18).The bands corresponding to those of proteins, nucleic acids, and carbohydrates were greatly reduced when compared to those from Figure 44. The energy of bands near 1670 cm^{-1}, 1412 cm^{-1}, and 1087 cm^{-1} were assigned to that of amide I, the symmetric stretches mode of a carboxylate group, and C-O polysaccharide group, respectively. A complete removal of proteins, nucleic acids, and carbohydrates were observed for biocide-treated stainless steel surfaces on day 6 (Figure 52) and day 12 (Figure 53). Only traces of carbohydrates remained attached to stainless steel surfaces in which Pseudomonas biofilms had developed for 18 days (Figure 54).

3.7. Decontamination of *B. subtilis* Spores from Selected Materials

3.7.1. Decontamination of Office Surface Materials. A wide range of office materials (e.g., woven carpet, acoustic ceiling tile, cork, concrete block, rough surface tile, smooth surface tile and wood floor tile) were contaminated with *B. subtilis* spores to simulate a biological attack

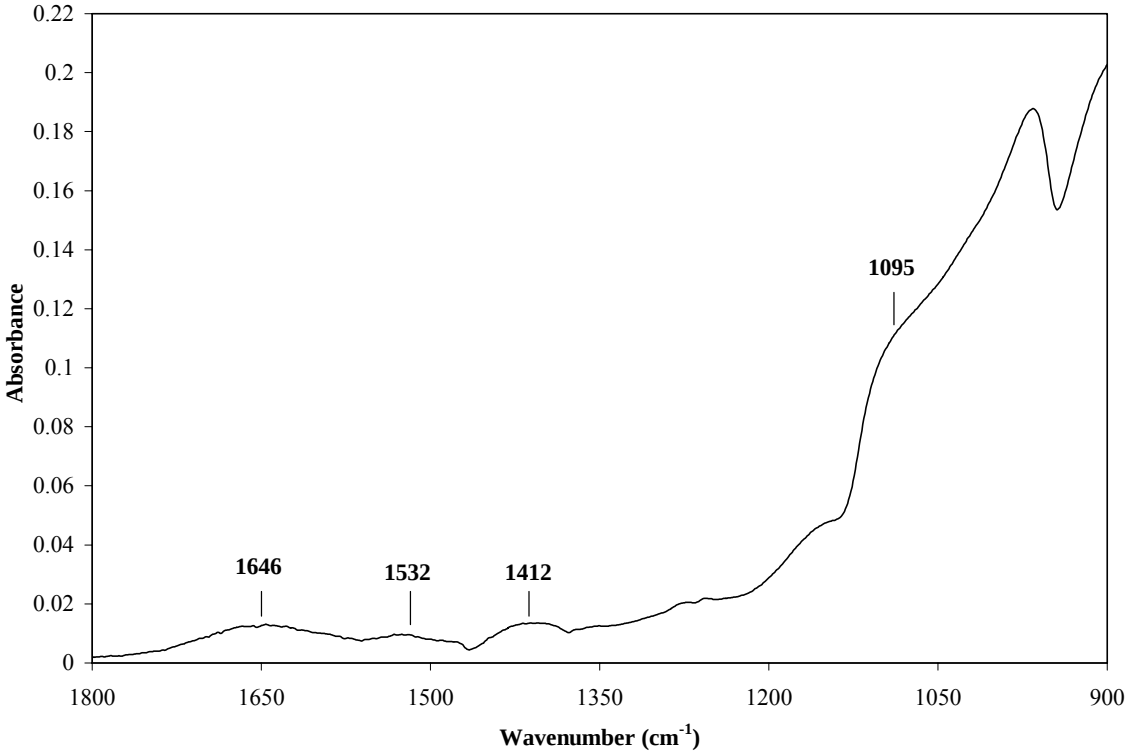


Figure 49. FTIR spectra of biocide-treated six-day old *P. aeruginosa* biofilm on aluminum. *P. aeruginosa* cells were grown in TSB and allowed to adhere to aluminum plates for 6 days. Biofilm was treated with biocidal complex (hydrogen peroxide: sodium hypochlorite) at a 1/10 ratio (2,500 ppm H_2O_2) for a 5 min contact time.

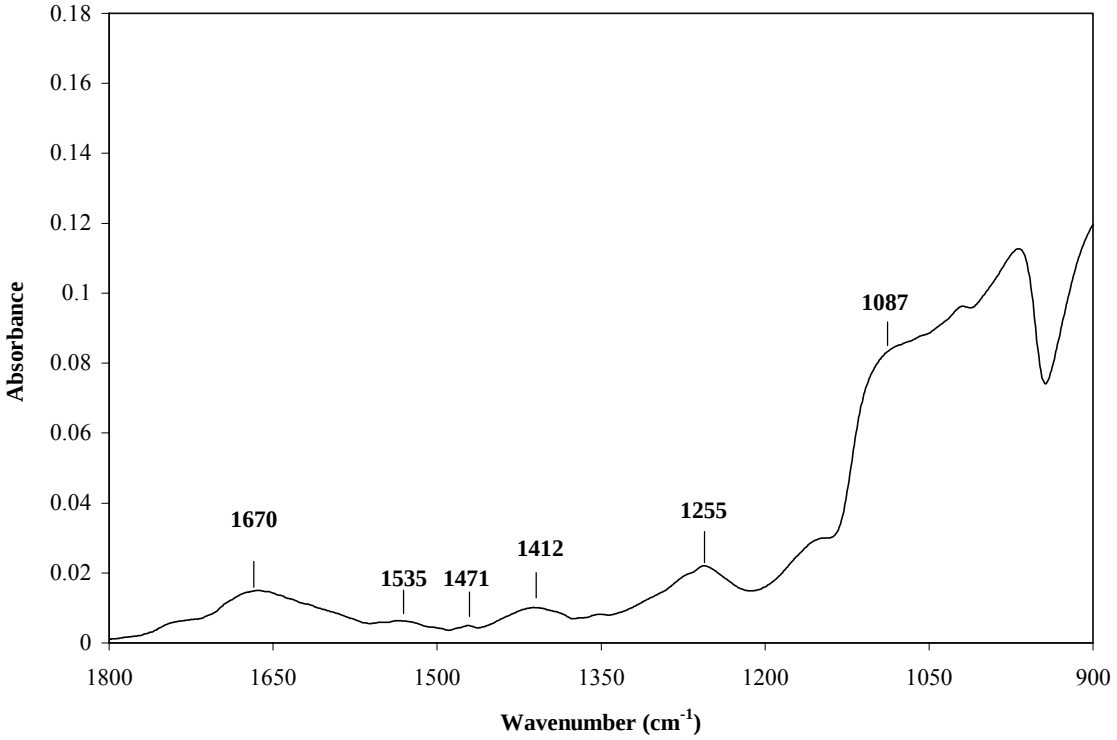


Figure 50. FTIR spectra of biocide-treated twelve-day old *P. aeruginosa* biofilm on aluminum. *P. aeruginosa* cells were grown in TSB and allowed to adhere to aluminum plates for 12 days. Biofilm was treated with biocidal complex (hydrogen peroxide: sodium hypochlorite) at a 1/10 ratio (2,500 ppm H_2O_2) for a 5 min contact time.

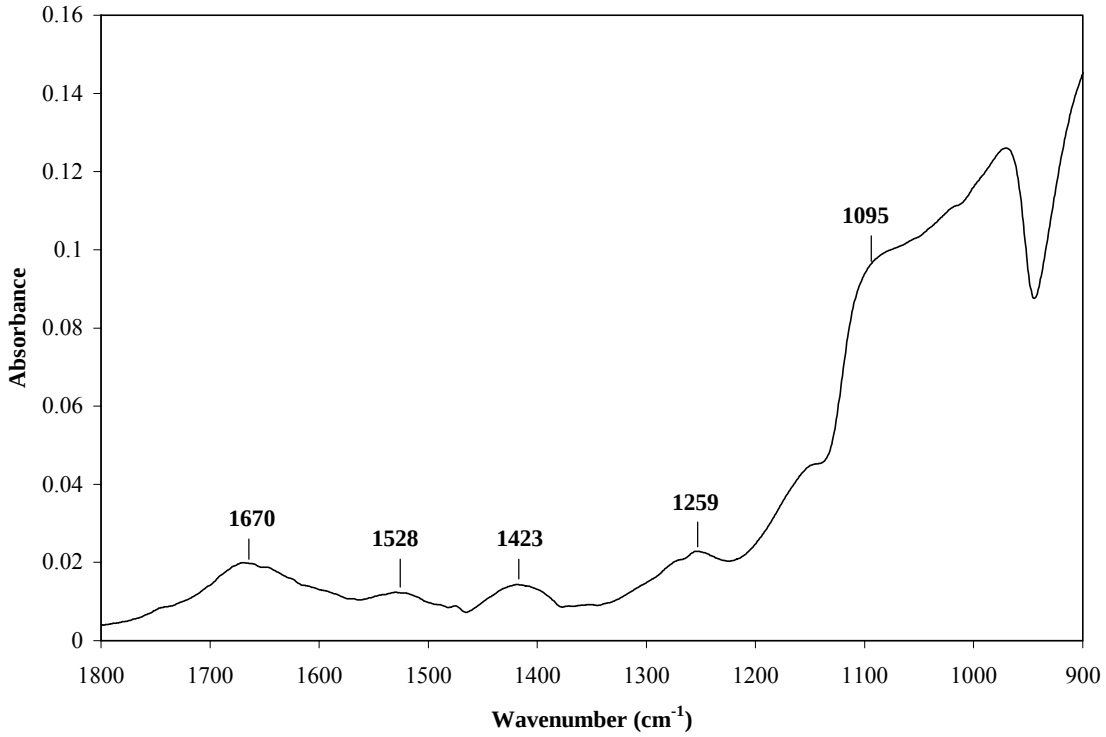


Figure 51. FTIR spectra of biocide-treated eighteen-day old *P. aeruginosa* biofilm on aluminum. *P. aeruginosa* cells were grown in TSB and allowed to adhere to aluminum plates for 18 days. Biofilm was treated with biocidal complex (hydrogen peroxide: sodium hypochlorite) at a 1/10 ratio (2,500 ppm H_2O_2) for a 5 min contact time.

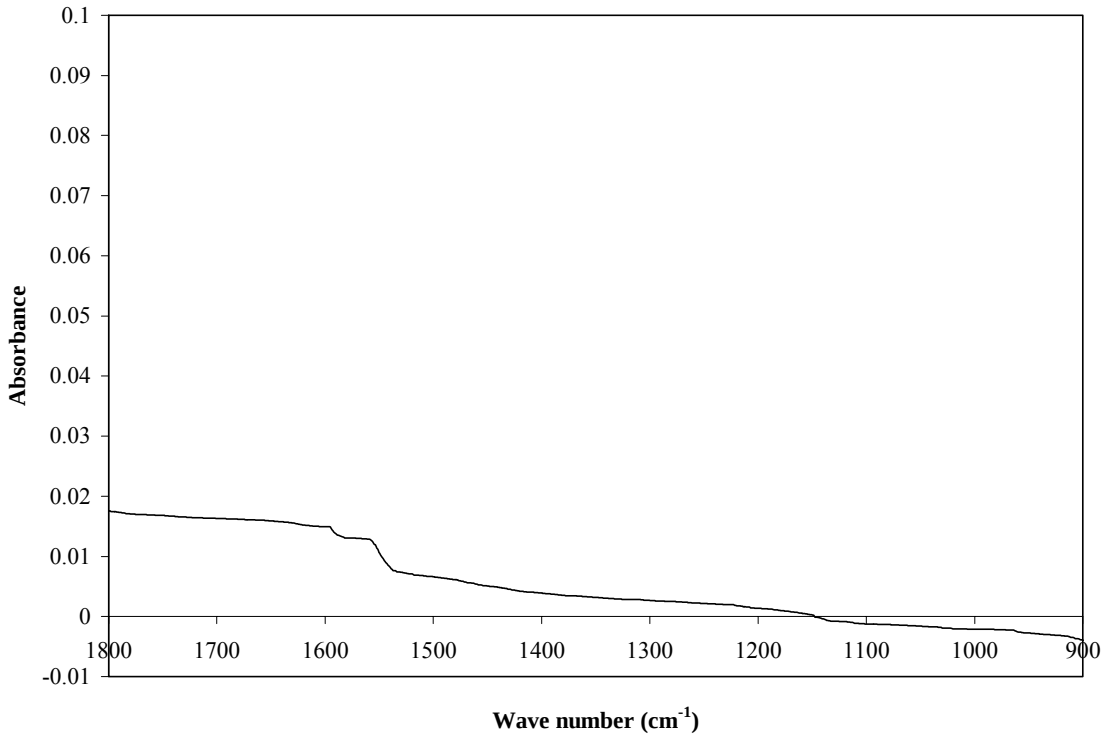


Figure 52. FTIR spectra of biocide-treated six-day old *P. aeruginosa* biofilm on stainless steel. *P. aeruginosa* cells were grown in TSB and allowed to adhere to aluminum plates for 6 days. Biofilm was treated with biocidal complex (hydrogen peroxide: sodium hypochlorite) at a 1/10 ratio (2,500 ppm H_2O_2) for a 5 min contact time.

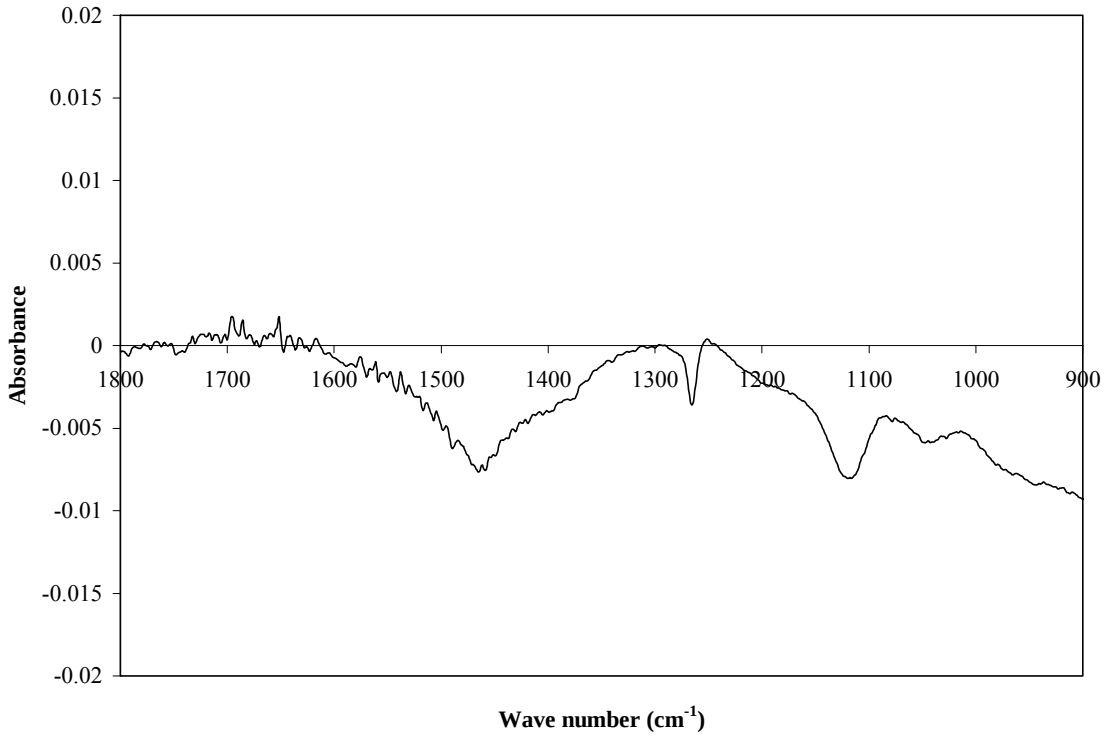


Figure 53. FTIR spectra of biocide-treated twelve-day old *P. aeruginosa* biofilm on stainless steel. *P. aeruginosa* cells were grown in TSB and allowed to adhere to aluminum plates for 12 days. Biofilm was treated with biocidal complex (hydrogen peroxide: sodium hypochlorite) at a 1/10 ratio (2,500 ppm H_2O_2) for a 5 min contact time.

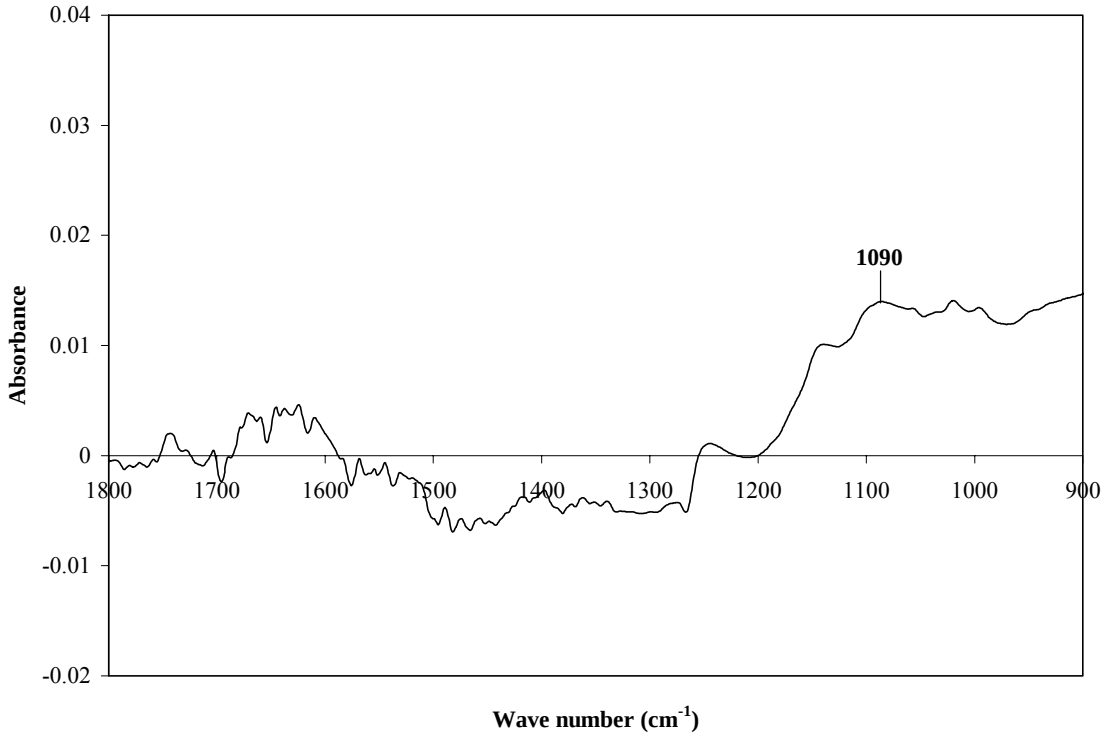


Figure 54. FTIR spectra of biocide-treated eighteen-day old *P. aeruginosa* biofilm on stainless steel. *P. aeruginosa* cells were grown in TSB and allowed to adhere to aluminum plates for 18 days. Biofilm was treated with biocidal complex (hydrogen peroxide: sodium hypochlorite) at a 1/10 ratio (2,500 ppm H_2O_2) for a 5 min contact time.

with *B. anthracis*. Sample panels were spray-treated with hydrogen peroxide, sodium hypochlorite or biocidal complex for a 5 or 10 min contact time. The concentrations tested were 2,500 ppm hydrogen peroxide, 25,000 ppm sodium hypochlorite or a combination of both with adjusted pH of 7. Contaminated panels averaged 5.8 to 6.7 log CFU in^{-2}. Results of the decontamination tests of woven carpet panels are summarized in Figure 55. Spore count reductions of 3.4 log and 4.3 log were observed for sodium hypochlorite and biocidal complex treatments, respectively. The difference was not significant ($P < 0.05$). A 10 min contact time with biocidal complex resulted in the complete kill of *B. subtilis* spores. Hydrogen peroxide treatments were not significantly ($P < 0.05$) different from untreated samples. Spore counts of treated acoustic ceiling tile panels were significantly reduced ($P < 0.05$) counts with biocidal complex after 5 min contact time (Figure 56). A 4.9 log reduction was observed after treatment with biocidal complex. However, no significant difference from control samples was observed with either hydrogen peroxide or sodium hypochlorite treatments. Similar findings were observed with treated spore-contaminated cork panels (Figure 57). Biocidal complex was the most effective sanitizer achieving a 4.5 log reduction after a 5 min contact time. Spore counts from hydrogen peroxide treated panels were not significantly ($P < 0.05$) less than control counts. Treatment with sodium hypochlorite resulted in a reduction of 2.2 log units. Decontamination data for treated concrete block panels are presented in Figure 58. Spore counts of sodium hypochlorite and biocidal complex treatments were significantly ($P < 0.05$) less than hydrogen peroxide and control. Sodium hypochlorite counts were significantly ($P < 0.05$) higher than biocidal complex counts. Spore count reductions of 4.1 log and 5.9 log were obtained for sodium hypochlorite and biocidal complex, respectively. Decontamination of rough surface tile panels revealed that biocidal complex was the most effective of the three sanitizers (Figure 59). Spore

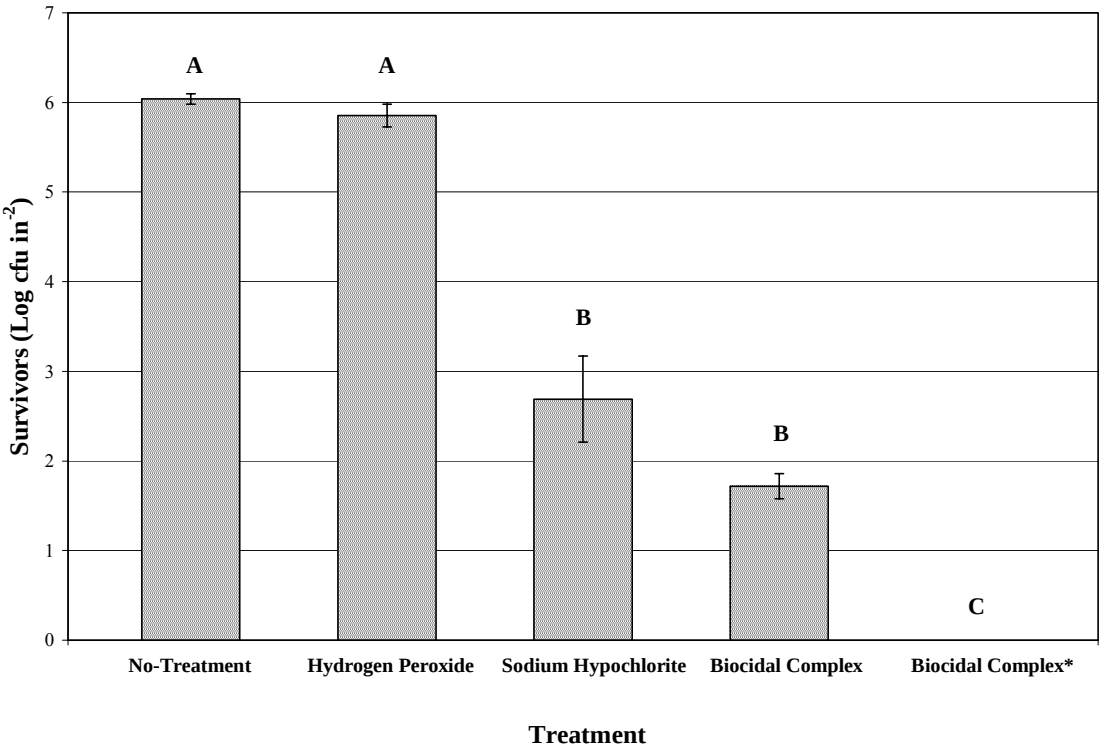


Figure 55. Disinfection of *B. subtilis* spore-contaminated woven carpet with biocides. All spore-contaminated carpet panels were treated with each biocide for a contact time of 5 min. * = woven carpet panels treated with biocidal complex for 10 min. Means with different superscripts indicate statistically significant differences ($P < 0.05$). Biocidal complex (hydrogen peroxide: sodium hypochlorite) (pH 7) was tested at a ratio of 1:10 (2,500 ppm H_2O_2). Error bars show the standard error of the mean.

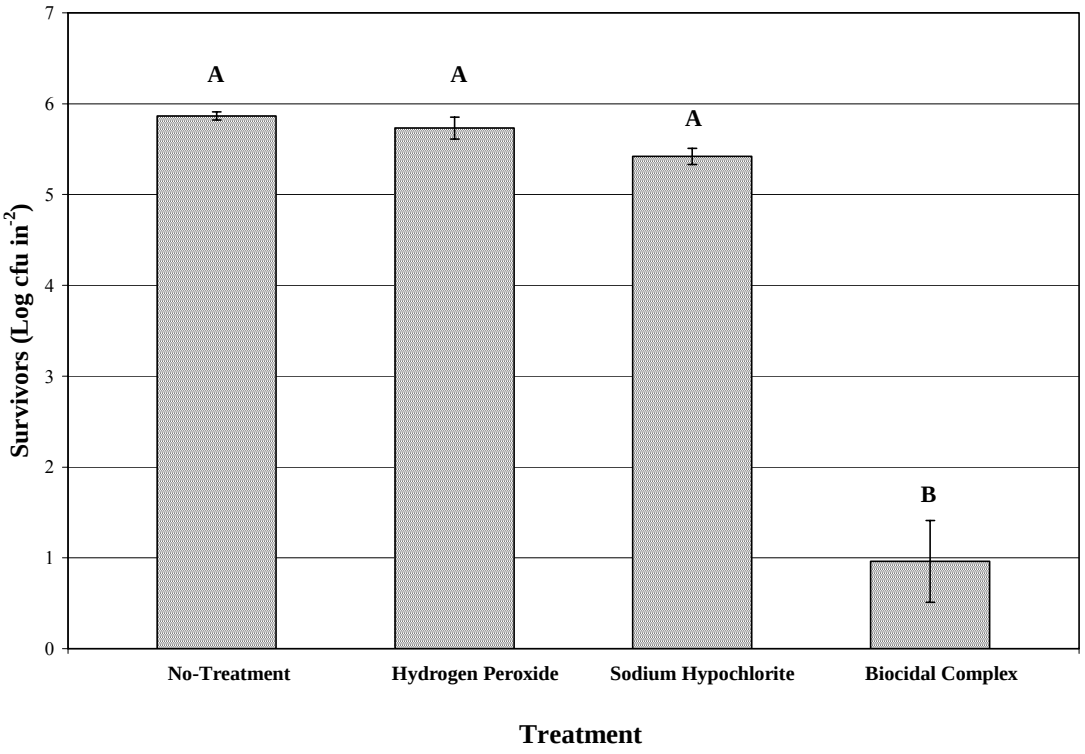


Figure 56. Disinfection of *B. subtilis* spore-contaminated acoustic ceiling tiles with biocides. All spore-contaminated acoustic ceiling tile panels were treated with each biocide for a contact time of 5 min. * = acoustic ceiling tile panels treated with biocidal complex for 10 min. Means with different superscripts indicate statistically significant differences ($P < 0.05$). Biocidal complex (hydrogen peroxide: sodium hypochlorite) (pH 7) was tested at a ratio of 1:10 (2,500 ppm H_2O_2). Error bars show the standard error of the mean.

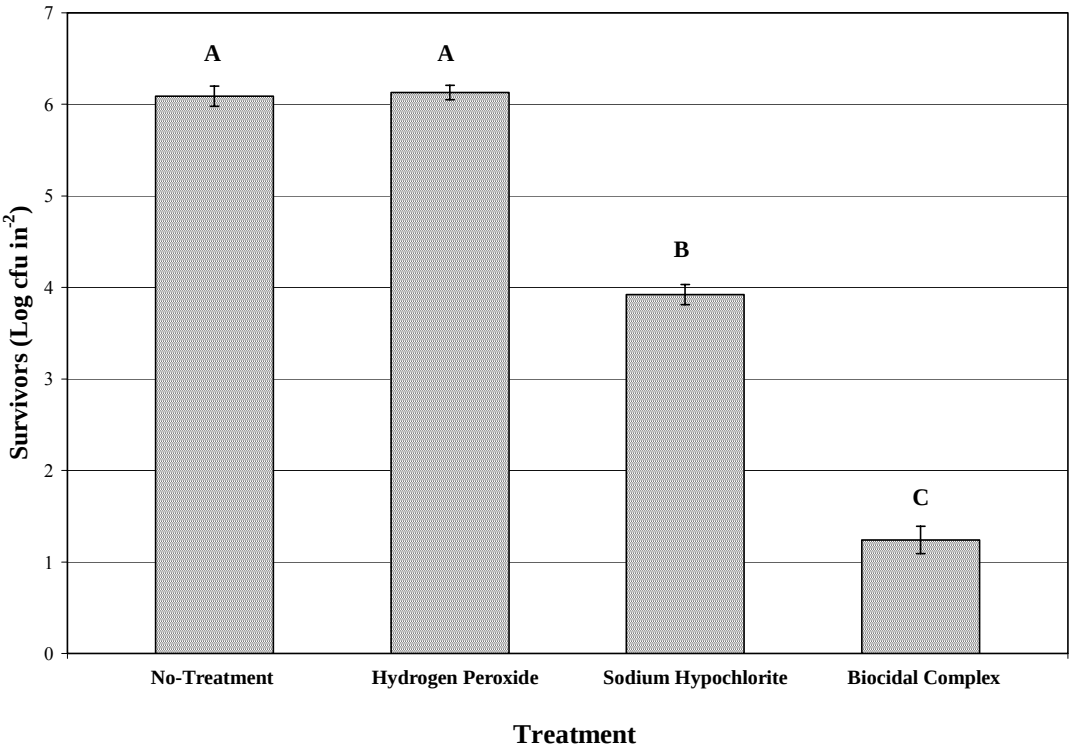


Figure 57. Disinfection of *B. subtilis* spore-contaminated cork samples with biocides. All spore-contaminated cork panels were treated with each biocide for a contact time of 5 min. * = cork panels treated with biocidal complex for 10 min. Means with different superscripts indicate statistically significant differences ($P < 0.05$ Biocidal complex (hydrogen peroxide: sodium hypochlorite) (pH 7) was tested at a ratio of 1:10 (2,500 ppm H_2O_2). Error bars show the standard error of the mean.

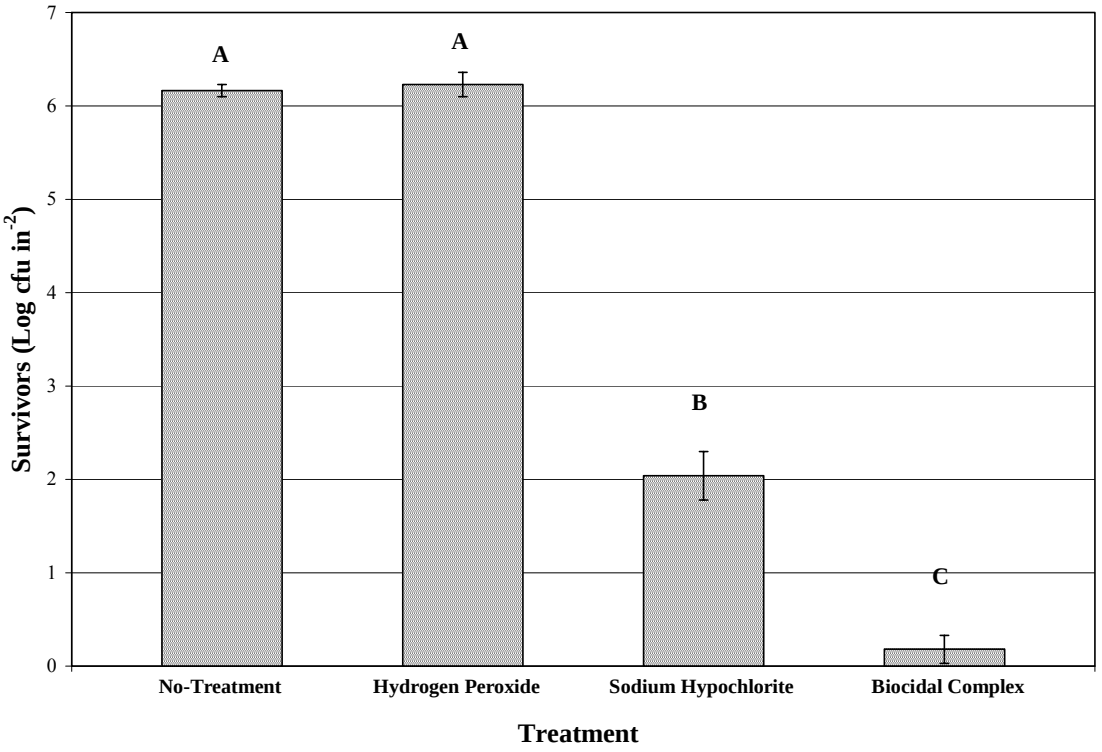


Figure 58. Disinfection of *B. subtilis* spore-contaminated concrete block with biocides. All spore-contaminated concrete block panels were treated with each biocide for a contact time of 5 min. * = concrete block panels treated with biocidal complex for 10 min. Means with different superscripts indicate statistically significant differences ($P < 0.05$). Biocidal complex (hydrogen peroxide: sodium hypochlorite) (pH 7) was tested at a ratio of 1:10 (2,500 ppm H_2O_2). Error bars show the standard error of the mean.

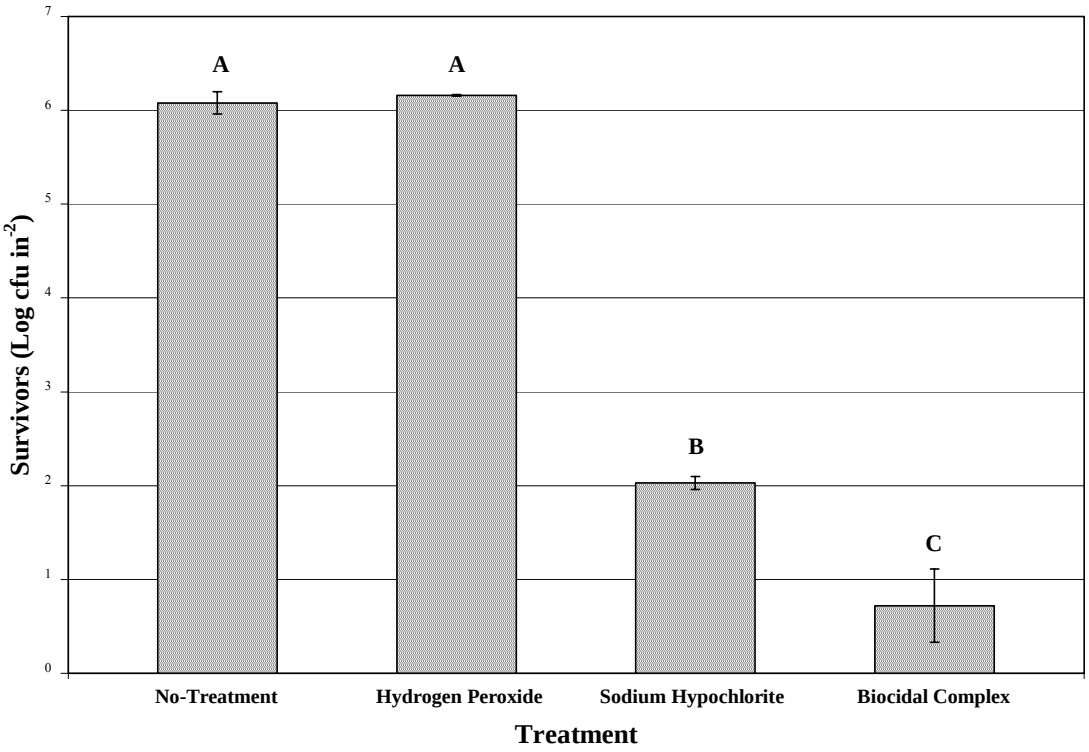


Figure 59. Disinfection of *B. subtilis* spore-contaminated rough surface tiles with biocides. All spore-contaminated rough surface tile panels were treated with each biocide for a contact time of 5 min. * = rough surface tile panels treated with biocidal complex for 10 min. Means with different superscripts indicate statistically significant differences ($P < 0.05$). Biocidal complex (hydrogen peroxide: sodium hypochlorite) (pH 7) was tested at a ratio of 1:10 (2,500 ppm H_2O_2). Error bars show the standard error of the mean.

counts were significantly ($P < 0.05$) reduced by 5.2 log units after 5 min treatment with biocidal complex. Only a 4.1 log reduction was observed for sodium hypochlorite treated samples, which was significant when compared only to hydrogen peroxide and control samples. Treated smooth surface tile panels resulted in spore count reductions of 4.4 log and 4.7 log for sodium hypochlorite and biocidal complex respectively (Figure 60). However, the difference between these treatments was not significant ($P < 0.05$). Nevertheless, 10 min treatments with biocidal complex resulted in the complete killing of spores; which was significantly different from 5 min treatments with sodium hypochlorite and biocidal complex. The results for wood floor tile panels are summarized in Figure 61. Significant ($P < 0.05$) differences from control samples were observed for all three sanitizers. Spore count reductions of 1.8 log, 4.2 log and 5.5 log were observed for hydrogen peroxide, sodium hypochlorite and biocidal complex after a 5 min contact time, respectively. Biocidal complex treatments for 10 min resulted in the complete killing of *B. subtilis* spores. These results were significantly different from 5 min treatments with sodium hypochlorite and biocidal complex. Overall, biocidal complex was the most effective biocide for decontaminating *B. subtilis* spore-contaminated selected office materials.

3.8. Effectiveness of Biocidal Complex at Sanderson Farms' Processing Facility

3.8.1. Decontamination of Poultry Carcasses. A study was conducted at the Laurel, MS poultry processing facility of Sanderson Farms. Eviscerated whole birds weighing an average of 7 lb were treated with three biocidal complex combinations (12.5 ppm: 125 ppm, 25 ppm: 250 ppm or 35 ppm: 350 ppm, for hydrogen peroxide and sodium hypochlorite respectively) trisodium phosphate (TSP) or chilled water for 1 ½ h to simulate a biocide-exposure time common to this processing facility. TSP and distilled water served as controls. The organisms targeted in this study were *E. coli* and Salmonella and total aerobic counts (TPC). Figure 62

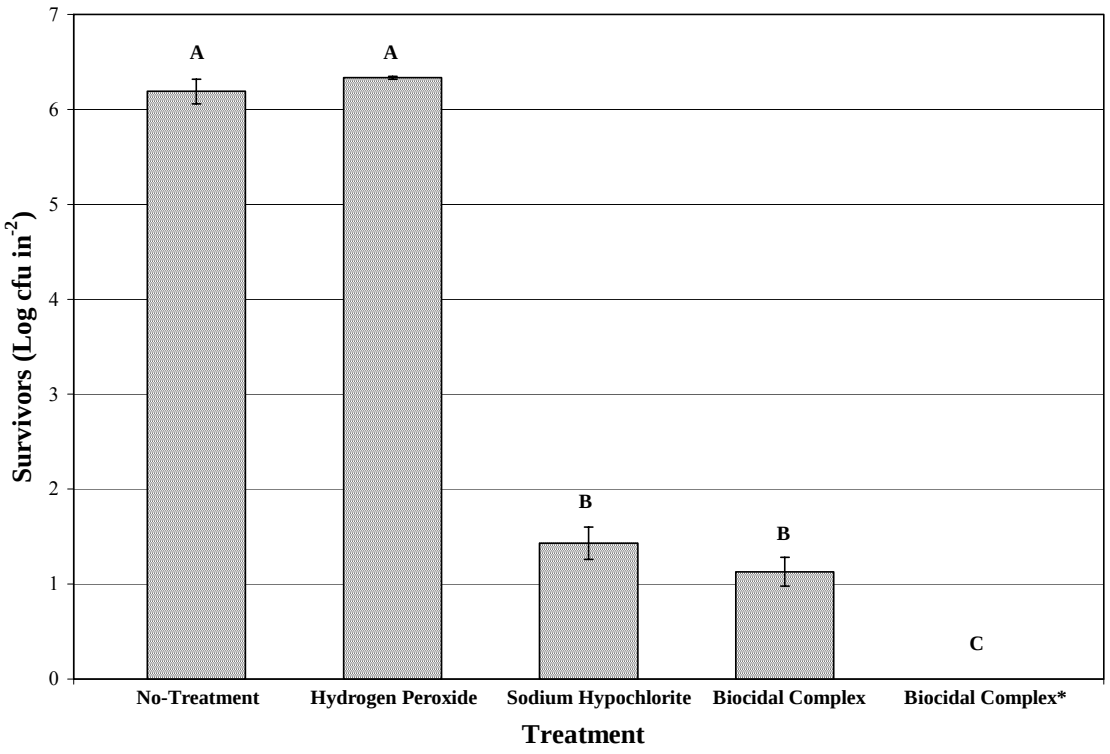


Figure 60. Disinfection of *B. subtilis* spore-contaminated smooth surface tiles with biocides. All spore-contaminated smooth surface tile panels were treated with each biocide for a contact time of 5 min. * = smooth surface tile panels treated with biocidal complex for 10 min. Means with different superscripts indicate statistically significant differences ($P < 0.05$). Biocidal complex (hydrogen peroxide: sodium hypochlorite) (pH 7) was tested at a ratio of 1:10 (2,500 ppm H_2O_2). Error bars show the standard error of the mean.

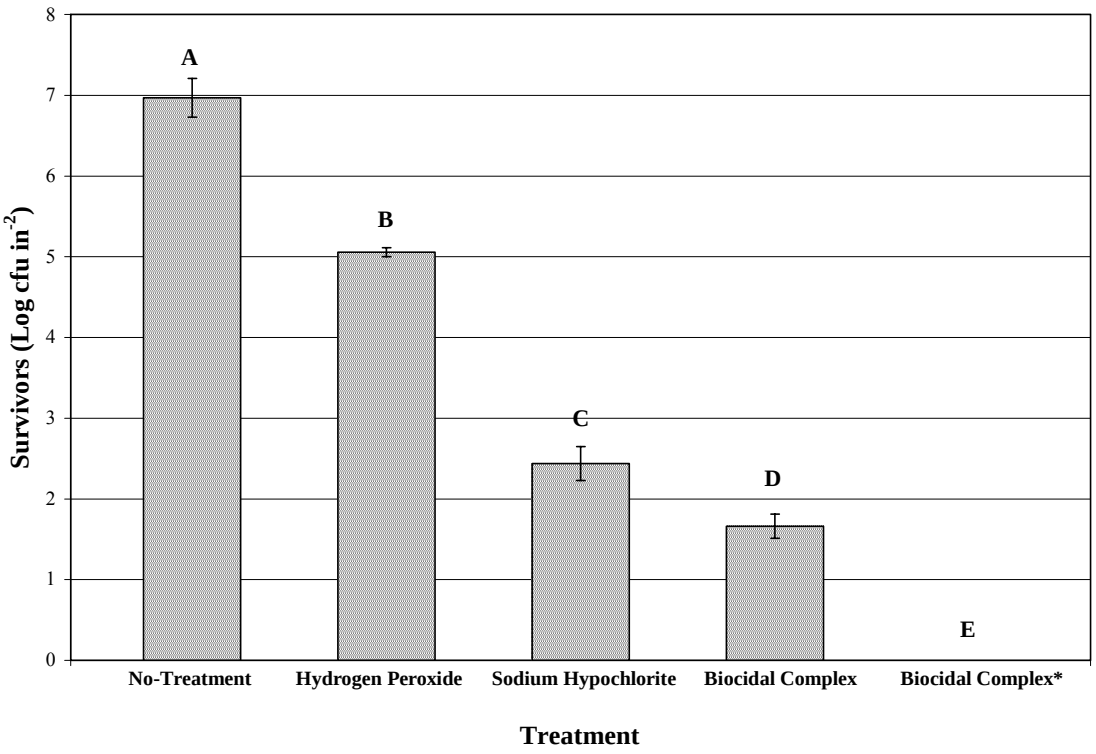


Figure 61. Disinfection of *B. subtilis* spore-contaminated wood floor tiles with biocides. All spore-contaminated wood floor tile panels were treated with each biocide for a contact time of 5 min. * = wood floor tile panels treated with biocidal complex for 10 min. Means with different superscripts indicate statistically significant differences ($P < 0.05$). Biocidal complex (hydrogen peroxide: sodium hypochlorite) (pH 7) was tested at a ratio of 1:10 (2,500 ppm H_2O_2). Error bars show the standard error of the mean.

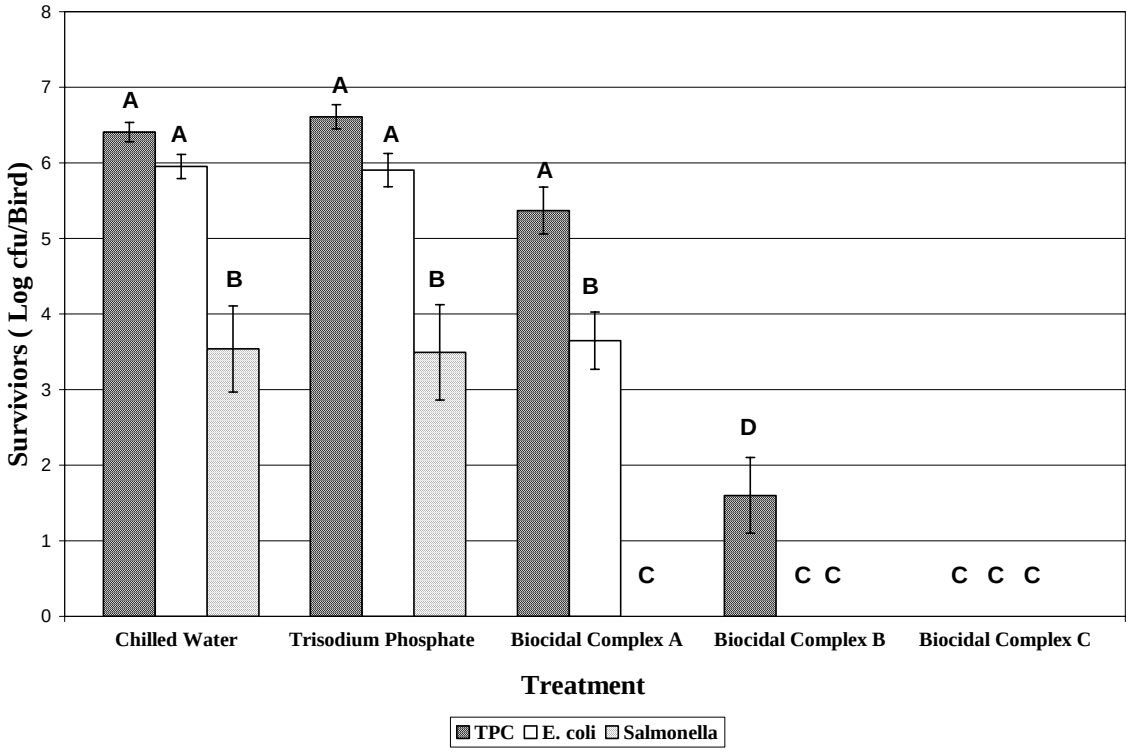


Figure 62. Decontamination of whole bird carcasses with biocides. Biocidal complex (hydrogen peroxide: sodium hypochlorite) solutions tested were (a) 12.5 ppm:125 ppm, (b) 25 ppm:250 ppm and (c) 35 ppm: 350 ppm. TPC = total plate counts. Means with different superscripts indicate statistically significant differences ($P < 0.05$). Error bars show the standard error of the mean.

summarizes the findings. Counts averaging 6.4, 6.0 and 3.5 log CFU per bird were obtained for total aerobic counts, *E. coli* and Salmonella, respectively. A significant ($P < 0.05$) difference was observed between TSP and biocidal complex treatments. However, TSP treatments were not significantly ($P < 0.05$) different from chilled water treatments. Biocidal complex treatment, at a concentration of 12.5 ppm: 125 ppm, reduced total aerobic counts by 1.0 log, which was not significantly different from chilled water or TSP treatments. Nevertheless, *E. coli* and Salmonella counts were significantly ($P < 0.05$) reduced by 2.3 log units and to undetectable levels, respectively. Treatments at a concentration of 25 ppm: 250 ppm also resulted in significant reduced counts. Total aerobic plate counts were reduced by 4.8 log units, where as *E. coli* and Salmonella counts were undetected. Similar findings were obtained for the highest biocidal complex concentration tested, in which *E. coli*, Salmonella and TPC remained undetected by the selective methods used in this study.

3.9. Decontamination of a Wash Table at Cora Texas Sugar Mill

3.9.1. Microbial Treatment of Wash Tables with Biocidal Complex. A section covered with a thick biofilm (~5mm) located behind the wash tables at Cora Texas sugar mill, LA was treated with biocidal complex, hydrogen peroxide or sodium hypochlorite. The biocide complex was applied at 5X, 20X and 50X the strength of 30 ppm: 200 ppm solution of hydrogen peroxide and sodium hypochlorite, respectively. Sterile distilled water, hydrogen peroxide (50X) and sodium hypochlorite (50X) were sprayed onto the test surface as controls. All treatments were sprayed for a volume of approximately 5.4 ml and sampled at 0.30 min, 15 min and 30 min contact times. The results are summarized in Figure 63. Total aerobic counts averaged 7.9 log in^{-2}. Biocidal complex solutions at 5X strength reduced total aerobic counts by 0.4 log, 3.5 log and 2.7 log after 0.3 min, 15 min and 30 min contact times, respectively. The increase in log survival at 30

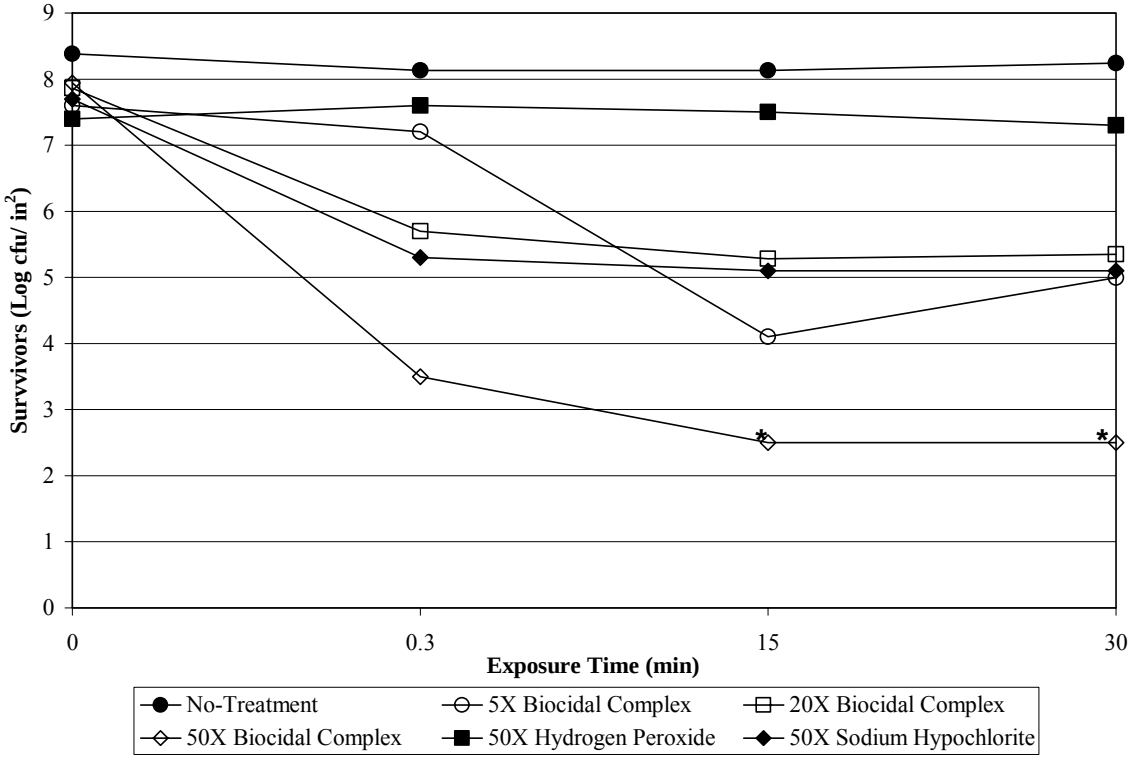


Figure 63. Decontamination of a biofilm covered wash table at Cora Texas sugar Mill, LA. Biocide complex was applied at 5X, 20X and 50X the strength of 30 ppm: 200 ppm hydrogen peroxide: sodium hypochlorite solution, respectively. Hydrogen peroxide or sodium hypochlorite controls were applied at 50X the strength of 30 ppm or 200 ppm, respectively. * = total aerobic plate counts were less than a 100 /ml.

min contact time could be explained by the washing off of biocidal complex from the test surface. Treatments with 20X biocidal complex resulted in a 2.2 log reduction after 0.30 min contact time. Nevertheless, log reductions at 15 and 30 min contact times did not differ much from 5X biocidal treatments. On the other hand, a cell count reduction of 4.4 log was observed at 0.30 min contact time with 50X biocidal complex treatment. Log reductions of total aerobic counts were higher than 5.4 log after 15 min and 30 min treatment with biocidal complex. Treatments with 50X hydrogen peroxide or 50X sodium hypochlorite were not as efficient sanitizers as 50X biocidal complex in reducing total aerobic counts. Hydrogen peroxide achieved only a 0.1 log reduction, where as sodium hypochlorite reduced counts by 2.6 log units after 30 min.

3.10. IB Biocide as a Hard Surface Disinfectant

3.10.1. Effectiveness of D/E Neutralizing Broth. D/E neutralizing broth was effective in neutralizing the antimicrobial activity of IB biocide. Growth was observed only in test tubes which received lower levels of inoculum within the range of 5-100 CFU/ml.

3.10.2. Effectiveness of IB Biocide as a Hard Surface Disinfectant against *B. subtilis* ATCC 19659 Spores. Pre-inoculated porcelain penicylinders averaged 1 x 10^6 *B. subtilis* spores/ carrier. Treatments of porcelain penicylinders with IB biocide for 10 min and 30 min resulted in 87% and 23% of carriers testing positive for the presence of *B. subtilis*. No spore survivors were detected after a 60 min treatment.

4. DISCUSSION

Chemical antimicrobials (i.e. disinfectants, sanitizers and sporicides) are used to control pathogenic microbial populations in the environment. They vary in their chemical structures and in their modes of action. However, the gross damage to microbes, exposed to lethal concentrations, may show considerable similarities.

Peroxygens, such as hydrogen peroxide and hypochlorites, such as sodium hypochlorite are examples of chemical antimicrobials widely accepted as disinfectants in many industries. Hydrogen peroxide is often used at concentrations ranging from 1-20% (Mullen, 2002). However, it can be corrosive to skin and metals. Sodium hypochlorite, commonly known as household bleach, is stored as a 5% solution often thickened with surfactants to provide good dirt penetration and prolong contact (Mullen, 2002). Because of their widespread use as disinfectants, hypochlorites serve as the standard for other disinfectants. Nevertheless, hypochlorites are rapidly inactivated by organic matter, release chlorine gas when mixed with acid or if the pH is below 5, and are corrosive (Mullen, 2002).

Synergism of antimicrobial activity between a mixture of hydrogen peroxide and sodium hypochlorite was confirmed against a wide variety of bacteria, both vegetative and spore forms. The biocidal "complex" needed from one sixth to one half the concentration of hydrogen peroxide and from one twentieth to one half that of sodium hypochlorite normally required to kill the test bacterial cells. In the case of bacterial spores (*Bacillus* sp.), MICs of biocidal complex ranged from one twentieth to one half and from one fourth to one half for hydrogen peroxide and sodium hypochlorite, respectively. Nevertheless, the concentrations needed to kill spores were still a 100 fold higher than for vegetative cells. Our findings agree with others in that bacterial spores are more resistant to antimicrobials than vegetative cells, therefore higher concentrations

and longer contact times are required for sporicidal compared to bactericidal action (Denyer and Maillard, 2002; Bloomfield and Arthur, 1995). FIC values for both bacterial cells and spores were less than one. According to Berembum, FIC values of less than one indicate that a synergistic effect exists between biocide components (Berenbaum, 1978).

The biocide "complex" is formed by adding hydrogen peroxide to sodium hypochlorite so that the weight ratio of the peroxide to the hypochlorite is about 1:10. A 1:10 ratio of hydrogen peroxide to sodium hypochlorite (4,000 ppm hydrogen peroxide) produced a 100% kill of *B. subtilis* spores suspended in distilled water after a 5 min contact time. Kills of 3%, 3%, 4% and 56% were observed for 1:1 (40, 000 hydrogen peroxide), 5:1 (50,000 hydrogen peroxide), 10:1 (40,000 ppm hydrogen peroxide) and 1:5 (10, 000 ppm hydrogen peroxide) ratios, respectively. The maximum effect was achieved with a 1:10 ratio.

A spectral scan (200 nm to 450 nm) of a 1:10 ratio mixture of hydrogen peroxide to sodium hypochlorite against a sodium hypochlorite baseline indicated the formation of a complex that disappeared after an extended period of time. The area below the curve of this spectral scan ($\int$Absorbance(λ)(d)λ = 55) closely resembles the area difference between the biocidal complex and sodium hypochlorite scans ($\int$Absorbance (λ)(d)λ = 74). A linear relationship was observed between the area below the curve for each scan and the percent kill of the biocidal complex. A 76% kill at a 5 min contact and an area of $\int$Absorbance(λ)(d) λ= 279 were obtained on day 0. The percent kill of the biocidal complex decreased by 15% and 47% when stored for 7 days and 42 days at 24˚C. The area below the curve for each scan was reduced by 4% and 7%. The chemical structure of this biocidal "complex" is uncertain but we postulate that it is a semi-stable complex, whose stability is disrupted by heat, acid, U/V exposure and the presence of organic matter (i.e., microbes). These are the same conditions that affect sodium

hypochlorite or hydrogen peroxide solutions (Fiorenza and Ward, 1997; Halliwell and Gutteridge, 1985; Nicoll and Smith, 1955; White 1999).

The antimicrobial activity and long term stability of the biocidal complex is a function of temperature. The antimicrobial activity was twice as high as that of sodium hypochlorite at 37˚C. Raising the temperature of the biocidal complex solution increased its rate of dissociation. However, the stability of the biocidal complex is less at higher temperatures and in the presence of U/V light. Storing a "biocide complex" solution at 4˚C in the absence of U/V light produced a half-life of 123 days. However, its half-life was reduced by 24% and 36% when stored at temperatures of 24˚C and 37˚C, respectively. A half-life of 93 days was observed for solutions stored in the presence of U/V light at 24˚C. Similar temperature and U/V light effects were observed for sodium hypochlorite or hydrogen peroxide solutions. The biocidal complex stability paralleled that of hydrogen peroxide, not sodium hypochlorite. At 4˚C, the half-life of hydrogen peroxide in the absence of U/V light was 220 days. Its half-life was reduced by 40% and 45% when stored at 24˚C and 37˚C. According to Nicoll and Smith (1955), an increase in temperature reduces the stability of dilute peroxide solutions. The rate of decomposition increases (for pure hydrogen peroxide solutions) about 2.4 fold for each 10˚C rise (Roth and Shanley, 1953). The half-life of sodium hypochlorite was 708 days at 4˚C and decreased by 75% and 76% when stored at 24˚C and 37˚C in the absence of U/V light, respectively. The half-lives for hydrogen peroxide and sodium hypochlorite solutions stored at 24˚C in the presence of light were 179 days and 132 days, respectively. Baker (1969) reported half-lives ranging between 60 and 1700 days for sodium hypochlorite solutions of 18 and 3%, respectively, at ambient temperatures. A decrease by a factor of six in sodium hypochlorite concentration resulted in almost a 30 fold increase in stability of the hypochlorite solution. Gordon et. al. (1997) reported similar findings

on sodium hypochlorite stability. In his study, 15%, 10% and 5% commercially produced sodium hypochlorite solutions were prepared and stored in the dark at 13˚C and at 27˚C for 28 days. As expected, higher reductions in the free available chlorine concentrations (FAC) for 15% and 10% solutions were observed during a 28 days trial than for a 5% solution. They estimated that the rate of decomposition of 10% or 15% solutions nearly doubles with every 12.2˚C temperature rise (White, 1999). The half-life of a 10-15% available chlorine solution will be also reduced three or four fold by U/V light exposure (White, 1999). A decrease in half-life by six fold has been observed in solutions of up to 20% (White, 1999).

The effectiveness of the biocidal complex depended on the pH of the solution. At a pH of 11, a 97% kill was observed for the biocidal complex solution compared to only 74% for a sodium hypochlorite solution. However, adjusting the pH from 11 to 6.5 yielded a 100% kill for both biocidal complex and sodium hypochlorite. The comparable effectiveness at the lower pH is explained by the fact that sodium hypochlorite activity is pH dependent. Hypochlorous acid, the most germicidal of all chlorine compounds, is formed upon the reaction of sodium hypochlorite and water (White, 1999). Hypochlorous acid is a weak acid that partially dissociates to form a hydrogen ion and hypochlorite ion. The hypochlorite ion is a poor disinfectant because of its inability to diffuse through the cell walls of microorganisms (White, 1999). At a pH above 9 and at 20˚C, 96% of the free available chlorine consists of hypochlorite ions compared to only 8% at a pH of 6.5 (White, 1999). Despite the effectiveness of sodium hypochlorite at a lower pH, commercial bleach solutions are stored at a pH 11 to preserve their stability (White, 1999). Long term storage of biocidal complex, sodium hypochlorite and hydrogen peroxide solutions were affected by acidic conditions as well. Half-lives of 680 days, 190 days and 160 days were observed for sodium hypochlorite, hydrogen peroxide and biocidal complex solutions at alkaline

or neutral pH. Adjusting the pH of each solution from pH 11 or 7 (for hydrogen peroxide) to 6.5 reduced their stability by 97%, 52% and 78%, respectively. Nicoll and Smith (1955) reported that the formation of radicals by the presence of various trace metals could explain the rapid decomposition of concentrated solutions of hydrogen peroxide in acid media. Other mechanisms include oxidation-reduction reactions (Barb et. al., 1949), the formation of unstable metal ion complexes (Glassner, 1950) and the decomposition of alkaline hydrogen peroxide in the presence of colloidal materials, particularly metals and large reactive surfaces (Nicoll and Smith, 1955). The use of distilled water has been reported to decrease the stability of hydrogen peroxide. According to Nicoll and Smith (1955), distilled water contains high catalytic impurities, particularly copper ions. They reported that copper sulfate at a concentration of 1 x 10^{-6} M had about the same effect on peroxide stability as distilled water. In addition to copper, ferrous (ic) ions can form unstable peroxides or complex per-ions which result in peroxide decomposition (Nicoll and Smith, 1955). Colloidal hydroxides can be formed as the alkali content of the solution increases, becoming a more active catalyst for hydrogen peroxide decomposition than the peroxides or complex per-ions (Nicoll and Smith, 1955). Nevertheless, ferric ions at concentrations of 10^{-6} and 10^{-5} M within the pH range 10.5 to 11.4 can act as stabilizers. Sodium hypochlorite stability can also be affected by the presence of trace metals. Iron in quantities as low as 0.5 mg/L will cause rapid deterioration of a 15% solution in just a few days (White, 1999). According to Gordon et. al. (1995), the maximum concentration of transition metal ions that will not significantly affect bleach decomposition is ~0.1 mg/L Ni^{2+} and ~1 mg/L Cu^{2+}. Addition of 1 mg/L of Cu^{2+} in a sodium hypochlorite solution caused a decreased of AFC by a factor of 1.4 during a 60 day period in the dark. The rate of hypochlorite decomposition was greatly enhanced with 1mg/L Ni^{2+}. No increase in the decomposition rate

was observed with the addition of 1 mg/L of Fe^{3+} and Mn^{2+}. However, ferric iron or manganese when present alone, behave as catalysts for sodium hypochlorite decomposition.

In the early 1960's, Seliger (1960) reported that a red-orange chemiluminescence was formed during the reaction of hydrogen peroxide and sodium hypochlorite. Work by Khan and Kasha (1963) and by Arnolg, Ogryzlo and Witzke (Kearns, 1971, Arnold et. al., 1965) confirmed that the chemiluminescence observed by Seliger was due to the presence of singlet oxygen. In the Spring of 1972, Allen and Stjernlholm (Maugh, 1973) presented evidence that electronically excited singlet oxygen acts as a microbicidal agent in phagocytosis. The antimicrobial activity of the "biocidal complex" is most likely a combination effect between oxidation and reductive mechanisms. A trans-1(2'- methoxyvinyl) pyrine (t-MVP) probe confirmed the presence of singlet oxygen in a 1:10 (2,500 ppm hydrogen peroxide) biocidal complex solution. An increase in singlet oxygen emission intensity of 72% and 6% was observed for hydrogen peroxide and biocidal complex solutions triggered with organic matter (*B. cereus* spores), respectively. It has been shown that Cu^{2+}, as a free ion or as part of a complex, generates singlet oxygen as the predominant reactive species upon reaction with hydrogen peroxide (Frelon et. al., 2003). Reports by Shiozawa (2000) confirmed the presence of other reactive oxygen species such as hydroxyl radicals, superoxide anion radicals and chlorine radicals in addition to singlet oxygen upon the reaction of hydrogen peroxide with sodium hypochlorite. Singlet oxygen and superoxide radicals react directly with moieties of high electron density such as carbon double bonds which are found in all major classes of biomolecules, leading to protein oxidation, non-enzymatic lipid peroxidation and nucleic acid (RNA and DNA) damage (Esterbauer et al., 1991and Kanner and Harel, 1985). Hydrogen peroxide is an oxidizing agent and can produce hydroxyl radicals in water (Block, 1983). Oxidative killing of metabolically active cells by

hydrogen peroxide is attributed to the generation of the hydroxyl radical (Shin et. al., 1994). Hydroxyl radicals are very small molecules and can easily diffuse through cell membranes damaging nucleic acids, proteins and lipids (Woznichak et. al., 2000, Juven an d Pierson, 1996). Chlorine containing sanitizers interact with enzymes containing acid groups to form weakly ionized compounds that inhibit cell metabolism but have little effect on cell morphology (Kim and Slavik, 1996). Hypochlorous acid reacts rapidly with proteins, DNA, lipids, thiols and disulfides (Noguchi et. al., 2002 and Hawkins et. al., 2003). The reaction of hypochlorous acid with DNA results in the generation of chloramines. Chloramines are toxic compounds that dissociate double stranded DNA due to disruption of hydrogen bonds (Hawkins et. al., 2003). Other studies have suggested that the reaction of hypochlorite with organic hydroperoxides results in the formation of radicals such as t-butyloxy radical but not singlet oxygen (Panasenko et. al., 1997). Organic hydroperoxides are present in certain amounts *in vivo* in cells and may be the intermediate compounds that react with hypochlorite leading to radical formation and initiation of lipid peroxidation.

The combination of hydrogen peroxide with peracetic acid has been found to be synergistic against Bacillus spores (Alasri et. al., 1992). Other combinations include hydrogen peroxide with sodium bicarbonate as a decontaminant of broiler carcasses (Fletcher et al., 1993) and with amino acids such as histidine, against *E. coli* cells (Brandi et al., 1991). Mixtures of 1.5% to 4% sodium hydroxide with 200 ppm sodium hypochlorite have been shown to be more sporicidal than either component used singly (Cousins and Allan, 1967). Methanol and other alcohols also show synergism when mixed with hypochlorite (Death and Coates, 1979). Our studies indicate that sodium hypochlorite is not only synergistic with hydrogen peroxide but with sodium peroxide as well. The MIC for the combined sodium peroxide and sodium hypochlorite

reaction was 50 ppm: 250 ppm, respectively, with an FIC of 0.83 against *P. aeruginosa* ATCC 19142 cells. The FIC value obtained for the sodium peroxide and sodium hypochlorite mixture was 0.03 units higher than the biocidal complex. The bactericidal effect of the sodium peroxide and sodium hypochlorite mixture was more bactericidal than either compound used singly.

4.1. As a Bactericide

Concerns for food safety have made consumers aware of food related diseases such as Salmonellosis that may be caused by the consumption of raw meat, poultry or other products contaminated with *Salmonella* sp. Salmonella is selected as a target pathogen because it is widespread in the environment (Murray, 1991) and it is one of the most common causes of foodborne illnesses. The cost worldwide on diseases reported in both humans and animals have mounted to several billions of dollars a year (Roberts, 1988). In the United States, an average of 76 million cases of foodborne illnesses (Mead et. al., 1999) and 40,000 cases of salmonellosis are reported each year, of which approximately 1,000 persons died each year of acute salmonellosis (National Center for Health Statistics, 2001).

The Food Safety and Inspection Service (FSIS), the USDA's agency responsible for ensuring the safety, wholesomeness, and accurate labeling of meat, poultry and egg products, issued on July 1996, the Pathogen Reduction (PR)-Hazard Analysis Critical Control Point (HACCP) system final rule (PR-HACCP rule) (FSIS, 1996). This rule (i) requires all meat and poultry establishments to develop and implement written sanitation operating procedures, (ii) requires meat and poultry slaughter establishments to conduct microbial testing of carcasses in order to verify their process controls for the prevention of fecal contamination, (iii) sets pathogen reduction performance standards for Salmonella prevalence for slaughter and raw product establishments, and (iv) requires all meat and poultry establishments to develop and implement a

system of preventive controls, known as HACCP (Hazard Analysis of Critical Control Points), to improve the safety of their products. Raw products currently covered by performance standards are carcasses of cows, bulls, steers and heifers, market hogs, broilers, ground turkey, chicken and ground beef. Targeting Salmonella in raw products may effectively reduce other foodborne pathogens of concern such as *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Listeria monocytogenes* Clostridium sp., Pseudomonas sp. and *Staphylococcus aureus* (Flowers, R. S., 1988).

In poultry processing, live birds are slaughtered, de-feathered, eviscerated, cleaned and chilled (Tsai et. al., 1991). During the chilling process, carcasses are immersed in large open tanks containing ice cold water and chemical disinfectants to control microbial populations and to improve the shelf life of finished products. Chlorine (as hypochlorous acid) has been the exclusive choice for many processors for its availability and low cost. Nevertheless, experimental reports have indicated that chlorine not only reacts with microorganisms but also with many inorganic and organic materials present in the chilled water thus reducing the availability of chlorine to act against microorganisms (Tsai et. al., 1991). Other compounds such as ozone, hydrogen peroxide, trisodium phosphate, chlorine dioxide and food acids are added to chilled water in some poultry processing plants. Nevertheless, these biocides have been found not to eliminate all Salmonella on poultry carcasses (Bryan and Doyle, 1995). In addition, poultry that enters the processing plants, carrying Salmonella, are a major source of final product contamination (Morris and Wells, 1970). Salmonella contamination can take place during transportation in which contaminated birds may shed Salmonella and contaminate transport containers (Bhatia and McNabb, 1980). Improper cleaning of transport containers may result in contamination of subsequent flocks. Another challenge arises when trying to eliminate

salmonella-infected surfaces protected by fecal material (wet or dry) or any other organic matter. Carr et. al. (1999) has shown that most disinfectants were ineffective against Salmonella in a field situation when this pathogen was protected by organic matter or existed in a biofilm.

The search for alternative biocides to reduce the numbers of potential food pathogens in food processing facilities continues. MICs for biocidal complex were significantly lower than equivalent MICs for sodium hypochlorite or hydrogen peroxide against *Salmonella typhimurium* ATCC 14028, *Escherichia coli* B ATCC 23226, *Campylobacter jejuni* ATCC 33560 and *Listeria monocytogenes*. On site studies at Sanderson Farms' poultry processing facility (Laurel, MS), revealed the effectiveness of biocidal complex solutions in reducing counts of Salmonella sp., *E. coli* and total aerobic counts on whole bird carcasses. Bacterial counts on trisodium phosphate (TSP) treatment, the biocide used by this processing facility, did not differ from control samples. The lowest biocidal complex concentration at a 1:10 ratio (12.5 ppm hydrogen peroxide) was significantly more effective than TSP in reducing counts of *E. coli* by 2.3 log CFU/ bird and Salmonella to undetectable levels. Both Salmonella and *E. coli* counts remained undetected at higher biocidal complex concentrations. Regulations set by FSIS on whole bird carcasses, as listed in Title 9 CFR part 381.94, indicate that *E. coli* levels should not exceed an upper limit of 1000 CFU/ml of rinse fluid or should be no more than 3 out of 13 carcasses testing positive for *E. coli* where 100 CFU/ml is the lower limit by the Most Probable Number (MPN) method. In addition, the percent of poultry samples testing positive for Salmonella should not exceed by 20% according to the standards set by FSIS.

4.2. As a Sporicide

The October 2001 bioterrorist attack with *Bacillus anthracis* spores in the United States awakened this country's necessity for strengthening its bio-defense system. In the wake of this

attack, a renew interest arise for studying new methods of bacterial spore inactivation as well as the mechanisms by which spores resist the lethal effect of various disinfection treatments (Nicholson and Galeano, 2003).

Bacillus anthracis is an aerobic, Gram- positive, sporulating bacillus and the causative agent of anthrax. There are three kinds of human anthrax: cutaneous, intestinal, and inhalation anthrax. Of all three, inhalation anthrax is highly fatal even after treatment with antibiotics (Atlas, 2002). The 2001 bioterrorist attack resulted in 22 confirmed cases of anthrax throughout the eastern United States (Dull et. al., 2003). There were 11 confirmed cases of inhalation anthrax and 7 confirmed cases of cutaneous anthrax, with additional 4 suspected cases of cutaneous anthrax (Bartlett et. al., 2002). The source of anthrax appeared to be 5 letters (all contaminated with the same *Bacillus anthracis* strain) sent through the US Postal Service to recipients in Florida, New York City and Washington, DC (Barlett et. al., 2002).One of the five contaminated letters was sent to Senator Tom Daschle which resulted in the contamination of his office located at the Hart Senate Office building in Washington, DC.

In an attempt to decontaminate Senator Tom Dashle's office and other contaminated rooms, chlorine dioxide at concentrations ranging from 500-800 ppm at 70-80 % relative humidity was applied for 12 to 20 h (USA Today, 2001). Chlorine dioxide is a toxic compound and can be used in its aqueous or gaseous state. The stability of gaseous chlorine dioxide is limited and a rapid decrease in concentration can be observed over time. Reports indicated that about 800 ppm chlorine dioxide gas quickly dropped to 16 ppm during the decontamination process of the Hart Senate Office building ("Anthrax Contamination of Hart Building Finished, 2001). Aqueous chlorine dioxide has been used as a disinfectant in the food industry and in

drinking water plants; however, the use of gaseous chlorine dioxide has been limited to pulp bleaching in the paper industry (Han et. al., 2002).

Experimental data supports the use of gaseous chlorine dioxide as a bactericidal agent for epoxy coated stainless steel surfaces (Han et. al., 1999) and as a sporicidal agent for paper and aluminum foil surfaces (Jeng and Woodworth, 1990). New findings by Han et. al. (2002) have indicated that spores of *Bacillus thuringiensis* (a *Bacillus anthracis* simulant) when present on envelope paper were more resistant to inactivation by chlorine dioxide gas than spore-contaminated wood, epoxy and plastic materials. A complete inactivation of *Bacillus thuringiensis* was achieved after exposure to 30 mg/L chlorine dioxide gas for 12 h under 85-92 % relative humidity. Other decontaminating technologies such as Nanotech (a microemulsion) from the University of Michigan and L-Gel (an oxidizing gel) from Lawerence Livermore Laboratory, CA have reported averaged reductions in *B. globigii* spore-contaminated surface materials of 6 and 4 log CFU in^{-2} after a 90 min and 30 min contact time, respectively (Harper and Larsen, 2001). Nevertheless, decontamination of a room containing materials such as rugs, carpets, clothes and computers still present numerous challenges ahead. Gluteraldehyde and peroxi compounds are active ingredients commonly used in liquid sterilization and high level disinfection (Block, 1991, Rutala, 1990). However, reports by Sagripanti and Bonifacino (1999) have indicated that commercial products containing these active ingredients had sporicidal activities different to those recommended for sterilization (10 and 11 h treatments) when applied to surfaces heavily contaminated with *Bacillus subtilis* spores. According to Sagripanti and Bonifacino (1999), general disinfectants (not specifically labeled for liquid sterilization) such as Cavicide (15.3 % isopropanol and 0.25% didisobutyl phenoxyethoxyethyl dimethyl benzylmammonium chloride), Lysol (7.24% o-benzyl-p-chlorophenol and 2.23% o-

phenylphenol), and chlorox (5.25% sodium hypochlorite) did not kill *B. subtilis* spores on contaminated devices.

The biocidal complex was tested against spores of four strains of the genus *Bacillus*. These included spores from *Bacillus subtilis*, *Bacillus pumilis* RJ 0055, *Bacillus cereus* and *Bacillus anthracis* (sterne strain). MICs of biocidal complex were found to be significantly lower than equivalent MICs for sodium hypochlorite or hydrogen peroxide against the vegetative cells and bacterial spores of the organisms listed above. Of the four bacterial strains tested, spores from *Bacillus subtilis* and *Bacillus cereus* appeared more resistant than the other two strains. Based on these observations, the effectiveness of the biocidal complex was tested against a wide selection of surface materials (e.g. carpet, ceramic tiles, acoustic tiles, concrete blocks, cork and wood floor tiles) pre-contaminated with *Bacillus subtilis* spores. The biocidal complex at a 1:10 ratio (2,500 ppm hydrogen peroxide, pH 7) was effective in reducing *B. subtilis* spore counts from 4 log CFU/in^2 to undetectable levels within a 5 or 10 min contact time. These results were shown to be significantly different from sodium hypochlorite or hydrogen peroxide treatments. According to EPA's test results for determining the sporicidal activity of disinfectants, IB biocide was an effective disinfectant of *B. subtilis* spore-contaminated porcelain carriers. No *Bacillus* spores were detected after a 60 min treatment.

4.3. As a Biofilm Remover

Biofilm formation is a step-wise process, which involves (1) conditioning of a solid surface by organic and inorganic material; (2) attachment of primary colonizing microorganisms; (3) growth and development of attached cells and (4) cell detachment from the biofilm surface (Lindsay et. al., 1996). Biofilms protect the attached bacteria from protozoan predators, desiccation, mechanical damage, antibiotics and sanitizers. Thus causing not only a considerably

hygiene risk in the food industry (Holah et al., 1989) but economic losses by technical failure in water systems, cooling towers, heat exchangers, chain lubrication systems and the like (Mattila-Sandholm and Wirtanen, 1992; Wirtanen et. al., 1996) as well as an increased risk of infection in the medical field due to growth on implants and other medical devices and impairing human health by causing caries (Mah and O'Toole, 2001). Subsequently, approaches to prevention, removal and killing of sessile and/or attached microorganisms are needed and sought in almost all fields of hygiene.

Biofilm formation in food processing environments has been widely investigated (Mattila-Sandholm and Wirtanen, 1992; Nelson, 1990). Studies carried out by Gibson et. al. (1995) on attached microorganisms in 17 different food processing environments reported that 79% of the isolates were Gram-negative rods, 8.6% Gram-positive cocci, 6.5% Gram-positive rods and 1.2% yeast strains. The most common isolates were *Pseudomonas*, *Staphylococcus* and *Enterobacter* sp. and they were characterized by a mucoid layer, an indication of EPS production. *Pseudomonas* are psychotrophic bacteria that attach to surface materials with relative ease and are the spoilage organisms of concern in chilled foods (Holah and Gibson, 2000). Coliforms are common Gram-negative bacteria found in the environment and are often associated with fecal matter. At times, coliforms can be isolated from surfaces of food processing equipment and/or final products thus serving as indicators of inadequate processing or post-processing contamination. The survival of spoilage bacteria and/or pathogens in the final product can result in reduced shelf life of the product as well as a potential hazard to human health.

Residues (organic and inorganic) remaining on surface materials after cleaning and disinfection can act as a conditioning layer thus allowing microorganisms to attached to this layer and later develop into a complex biofilm community (Mettler and Carpentier, 1998).

Stainless steel is a material commonly used in the food industry (Lewis and Gilmour, 1987). Bioadhesion to steel surfaces can lead to contamination or deterioration of foods resulting in high economic losses to the industry (Bott, 1990). Other commonly found surfaces that support the growth of foodborne pathogens and spoilage bacteria as biofilms include aluminum, glass, BunaN, Teflon seals and nylon materials (Kumar and Anand, 1998). Adhesion to surface materials is not only affected by the presence of a conditioning layer but also by the nature and degree of roughness of the substratum, temperature, pH, nutrient and time availability.

Microbial counts of on site biofilms at Cora Texas sugar mill and "synthetic" *P. aeruginosa* ATCC 19142 biofilms were greatly reduced or eliminated upon treatment with biocidal complex. Biofilms at Cora Texas showed >5.4 log reduction in microbial counts after treatments with biocidal complex. Biocidal complex at a 1:10 ratio (2,500 ppm hydrogen peroxide) reduced counts of *Pseudomonas aeruginosa* ATCC 19142 biofilms on both aluminum and stainless steel surfaces. Cell numbers were significantly reduced ($P < 0.05$) after 1 min exposure to biocidal complex, resulting in 3.7 and 5.7 log CFU in^{-2} reductions, whereas treatments with sodium hypochlorite resulted in reductions of 2.1 and 3.1 log CFU in^{-2} for aluminum and stainless steel plates, respectively. No cells could be recovered after 20 min treatment with biocidal complex on either surface. Cell reductions of 4.9 and 4.8 log CFU in^{-2} were observed for sodium hypochlorite on both aluminum and stainless steel surfaces, respectively. No significant reductions in cell numbers were observed for hydrogen peroxide (2,500 ppm). Guidelines established by the AOAC Germicidal and Detergent Sanitizer Test state that decreases in attached cell numbers by ≥ 3 log units would be an acceptable target value for sanitizer efficacy against biofilm cells (Lindsay et. al., 2002; Lindsay and von Holy, 1999 and Mosteller and Bishop, 1993). Hypochlorites and the active compound hypochlorous acid

penetrate biofilms poorly due to the neutralization of chlorine as it reacts faster with organic materials found on the surface of biofilms than it can diffuse into the biofilm interior (Stewart *et. al.* 2001;Chen and Stewart 1996; Xu *et. al.* 1996). Reduced counts of 1.1 log and 0.4 log after 60 min treatment of six days old *Pseudomonas aeruginosa* and *Klebsiella pneumonaie* biofilms with 1000 mg L^{-1} chlorosulfamate and alkaline sodium hypochlorite have been reported, respectively (Stewart et. al., 2001). Similar penetration effects due to a reaction-diffusion interaction have been observed for hydrogen peroxide in *Pseudomonas aeruginosa* biofilms (Stewart et. al. 2000).

SEM and TEM images of biocidal complex-treated attached *Pseudomonas aeruginosa* cells revealed roughness, indentations and perforations to cell walls and membranes as well as an increase in cell length, corresponding to high kill figures obtained for this sanitizer after 1 min (99.9% and 99.999%), 5 min (99.9999% and 99.9999%) and 20 min (99.999999% and 99.999999%) contact time on aluminum and stainless steel surfaces, respectively. The morphological changes observed on cell surfaces after treatment with biocidal complex can be attributed to radical formation and cell membrane permeability. Similar surface changes have been observed in planktonic and sessile *B. subtilis* and *P. fluoresecens* cells after treatment with a mixture of hydrogen peroxide and peracetic acid (Lindsay and von Holy, 1999). The hydrogen peroxide and peracetic acid combination acts by disrupting the sulfhydryl and sulphur bonds within enzymes, leading to disruption of cell membrane components. They also disrupt the chemiosmotic function of the membrane transport system by damaging the cell wall (Gougeon et. al., 1996). The increase in cell length as an indication of cell damage is also supported by CSLM images. CSLM images revealed that cells stained red, indicating cell death, after treatment of biofilms with biocidal complex on both aluminum and stainless steel surfaces.

However, other .oxidizing agents such as chlrorine dioxide containing sanitizers also increase lengths of *Pseudomonas fluorescens* M2 cells in biofilms, but CSLM images. revealed that 90% of the cells stained yellow, an indication of cell injury, and did not stain red, an indication of cell death (Lindsay et. al., 2002) It is important to take into account injured cell populations during sanitizer treatments as such populations may later recover and re-colonize surfaces. These changes and increased cell length were not observed for control cells which received the same sample preparation as biocidal complex-treated cells.

Sessile *P. aeruginosa* cells attached to stainless steel were more susceptible to treatment with biocidal complex than cells attached to aluminum surfaces. These results agree with other findings that sanitizer efficacy against attached cells also depends on the type of surface material (Frank and Chimielewski, 1997). *Pseudomonas aeruginosa* biofilms appeared thicker on aluminum than stainless steel surfaces. Therefore, the difference in EPS composition observed on aluminum and stainless steel surfaces may have influenced sanitizer's efficacy. Reports by Vandevivere and Kirchman (1993) have indicated that the amount and composition of EPS produced by bacteria is dependent on the type of attachment surface. This difference in EPS composition was also observed with FTIR spectrometry. The changes observed in the spectra taken during the development and maturation of Pseudomonas biofilm (day 6 to day 18) suggest changes in chemical bonding with surface atoms involving COO^- and NH_3^+ interactions (Cheung et. al., 2000). These results also agree with findings made by Fletcher and Marshall (1982), who suggested that proteins often function as adhesins in specific attachment mechanisms. It is also possible that functional groups of a given polymer may act in adhesion at different times depending on environmental conditions, medium compositions and substratum chemistries (Paul and Jeffrey, 1985)

Techniques such as physical scrubbing of surfaces and the use of high pressure water sprays are often applied in food processing plants for the removal of bacterial biofilms (Gibson et. al., 1999). A downside to their use is their ability to generate aerosols which results in the dispersion of surviving microorganisms over an extensive area (Holah et. al., 1993). Methods such as electric fields (Blenkinsopp et. al., 1992) and ultrasound (Mott et. al., 1998) have been used to enhance the removal of biofilms as well as the efficacy of biocides against biofilms. Detergents are also used in food processing environments but their formulations often focus in the removal of particular type of food soils rather than biofilms (Holah and Gibson, 2000). An exception is EDTA, which has been reported to improve the removal of *Bacillus* biofilms from soiled and unsoiled surfaces (Wirtanen et. al. 1996). The biocidal complex was not only capable of penetrating and effectively killing *Pseudomonas aeruginosa* cells embedded within biofilms but also removed biofilms from aluminum and stainless steel surfaces. Observations of biofilm changes upon exposure to biocidal complex were monitored using FTIR spectrophotometry and CSLM. FTIR spectra of six, twelve and eighteen days old *P. aeruginosa* biofilms treated with biocidal complex for 5 min revealed a drastic reduction or complete removal of proteins, nucleic acids and carbohydrates from both aluminum and stainless steel surfaces. Similar findings were observed with CSLM. CSLM images of six days old *P. aeruginosa* biofilms treated with biocidal complex showed not only the killing of cells within 1 min contact time but the disappearance of cells from stainless steel and aluminum surfaces after a 5 min and 20 min contact time, respectively. Chemical compounds such as peracetic acid, mercuric chloride and formaldehyde have been found to have no effect on biofilms (Carpentier and Cerf, 1993). Oxidizing substances such as chlorine or peroxoacetic acid are often used in the removal of biofilms (Meyer, 2003). However, their activity is reduced in the presence of organic matter

This study has shown a unique biocide composition that is formed from a hydrogen peroxide and sodium hypochlorite mixture, where a semi-stable biocidal complex is formed by adding the peroxide to the hypochlorite in an amount so that the weight ratio of the peroxide to the hypochlorite is no less than 1:10.The activity of the biocide is effective for at least 42 days at an alkaline pH stored in the dark at 4˚C. It is pH independent, non-corrosive and can be effective in both a dip and spray mode. The biocidal complex is synergistic against a range of Gram-positive, Gram-negative and bacterial spores present in their planktonic or sessile state. The use of this biocidal complex may provide a safe, effective and easy method for killing potential pathogens as well as for disinfecting and removing biofilms, as they pose a threat to human safety, particularly in the Food Industry.

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VITA

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